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Dr. J. T. EDWARDS

It is with deep sorrow that the Editors have to record the death of their esteemed colleague and co-editor, Dr. J. T. Edwards.

Readers will recall that in 1940 the late Sir John M'Fadyean decided to suspend issues of the Journal for the period of the war: before this emergency arose, however, he had made known to Dr. Edwards his wishes regarding its publication in the future.

Through Dr. Edwards' inspiration and enthusiasm, the Journal was revived in 1943 and its successful resumption was due, in no small measure, to his labours. It is fitting that the debt which is owing to him should be recorded.

He was a man of rare literary ability who never spared himself in what he regarded as a duty to his fellows. With his distinguished record as a research worker and wide experience at home and abroad, he was eminently competent to judge the contributions of others and he did all in his power to ensure that the Journal was produced in a manner worthy of its Founder.

THE DISTRIBUTION OF SULPHONE* IN THE TISSUES OF VARIOUS ANIMALS

By

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INTRODUCTION

At an early stage of the work on the treatment of leprosy with sulphone it was thought that very high concentrations were produced in the skin (Cochrane, Ramaniyam, Paul and Russel, 1949). This is now known to be untrue, although the concentration in the skin and some organs may be higher than in the blood (Simpson and Molesworth, 1950).

The distribution of sulphone in various tissues was investigated because of its importance in the treatment of leprosy (Francis and Spinks, 1950), and possibly in Johne's disease. The work brought to light differences in distribution between animals of different species and ages, which may explain varying toxicities in different animals.

METHODS

Tissues were extracted by grinding in 5 per cent trichloroacetic acid to give a 10 per cent suspension. After the debris had settled, 0·5 ml. of supernatant was taken and 0·25 ml. of 5 per cent trichloroacetic acid added. The estimations were then performed according to the method of Rose and Bevan (1944).

RESULTS

Mice. The results of the first small series of experiments, in which sulphadiazine was included for comparison, are shown in Table I. With this drug the tissue concentrations were not appreciably higher than the blood concentrations, and the skin concentration was much lower. With sulphone the concentrations in the liver were relatively higher. Similar results were obtained in mice infected with the vole acid-fast bacillus in which the lungs are an almost solid mass of lesions consisting of little but acid-fast bacilli.

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*4:4′ diaminodiphenyl sulphone.

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TABLE I.

BLOOD AND TISSUE CONCENTRATIONS OF SULPHONE AND SULPHADIAZINE IN MICE KILLED THREE HOURS AFTER TWO OR THREE DOSES OF THE RESPECTIVE DRUGS

			Concentration of Drug—mg./100 ml. or g.			
			Liver	*Lung*	*Skin*	*Blood*
Mice given 8 mg. sulphadiazine per os twice daily	Normal mice	Mice killed 3 hours after the second dose of sulphadiazine	9·2 10·8	10·0 12·8	6·8 7·0	10·8 11·6
		Average	10·0	11·4	6·9	11·2
Mice given 2 mg. sulphone per os twice daily	Normal mice	Mice killed 3 hours after the second dose of sulphone	4·0 5·2	2·8 3·8	2·3 3·2	3·0 3·8
		Average	4·6	3·3	2·8	3·4
	Mice infected with the vole acid-fast bacillus	Mice killed 3 hours after the second dose of sulphone	3·2 2·2 2·8	1·8 2·4 2·4	2·0 2·0 2·0	2·0 2·5 2·0
		Mice killed 3 hours after the third dose of sulphone	3·2 2·4 2·0	1·6 1·6 1·0	1·8 1·4 1·0	1·6 1·8 1·0
		Average	2·6	1·8	1·7	1·8

Pigs. Pigs were injected intraperitoneally with 0·25 g. of sulphone per kg. The average concentration of sulphone in the blood and skin was 3·6 mg/100 ml. on the first day after injection and 2·9 and 2·8 mg/100 ml. respectively on the second day. The pigs were killed on the third day and the results of various estimations are shown in Table II. It will be seen that the concentration in the liver was appreciably higher than in the blood.

TABLE II

BLOOD AND TISSUE CONCENTRATIONS OF SULPHONE IN PIGS GIVEN AN INTRAPERITONEAL DOSE OF 0·25 GM./KG., SUSPENDED IN LIQUID PARAFFIN*

	Concentration of Drug—mg./100 ml. or gm.			
	Liver	*Lung*	*Skin*	*Blood*
Concentration of sulphone in various tissues at the time of death	4·0 3·24	2·8 1·8	1·8 1·8	2·4 1·5
Average	3·62	2·3	1·8	1·95

*The pigs were killed on the third day.

Dogs. One dog was given 0·1 mg. of sulphone per kg. twice daily. A series of 7 blood and 7 skin samples was examined. In taking biopsy samples cocaine was used as a local anaesthetic and about 0·2g. of skin was removed. The average concentration for both blood and skin was 1·44 mg/100 ml. In one dog given 0·2 g./kg. of sulphone subcutaneously and killed 48 hours later the following concentrations were obtained.

Blood, 1·0; lung, 1·6; liver, 2·0 ; spleen, 1·1; skin, 1·3; small intestine (wall), 1·46; duodenum (wall), 1·3; caecum (wall) 2·44.

Cows. Two non-lactating cows that were being killed were injected with 40 g. of sulphone (about 100 mg./kg.) as "Udolac"*; approximately 13g. were injected into each of three quarters. Two days later the blood concentration was 3·6 mg./100 ml. in cow 229 and 2·8 mg./100 ml. in cow 231. The cows were killed three days after the injection and the results are shown in Table III, together with those in a calf that was dosed orally with 200 mg. of sulphone per kg. The concentrations in the injected quarters were considerably lower than are usually obtained (Francis; 1947, 1949). It will be noted that the concentrations in the uninjected quarter and in the right supramammary lymph-node were no higher than in some other tissues. The results in cattle were of special interest because it will be seen that, unlike those in the mouse, dog and pig, the tissue concentrations, even of the liver, were lower than the blood concentrations: moreover, it is known that sulphone is less toxic for the bovine than for other animals studied.

Sheep. The sheep is particularly susceptible to the toxic effects of sulphone on the nervous system, and to a lesser extent of sulphanilamide (Francis; 1947, 1949; Francis and Spinks, 1950). Two sheep were given an initial dose of 50 mg. of sulphone per kg. and 100 mg. 24 hours later. Sheep 248 was killed 24 hours after the second dose, but sheep 249 was killed three hours after receiving the second dose of sulphone because it was showing nervous symptoms. It will be observed that the tissue concentrations in sheep 249 were lower than in sheep 248 (Table III). However, the average blood concentration in the two sheep was 1.6 mg./100 ml. and brain concentration 1·2 mg./100 ml., whereas in the two cows the concentrations were 2·1 and 0·15 respectively. The concentration in the brain of the calf was much higher than in the cows.

Mice. Small mice are more liable to develop nervous symptoms when given sulphone than are large mice. Mice weighing 15 to 17 g., and 25 to 35 g. were given food containing 0·1 per cent of sulphone for five days and estimations were carried out on the blood, liver and brain. Nineteen large and twelve small mice were used.

*Trade mark of Imperial (Pharmaceutical) Limited. It is a 33·3 per cent suspension of sulphone in a paraffin base.

TABLE III.

BLOOD AND TISSUE CONCENTRATIONS IN SHEEP AND COWS FOLLOWING THE ADMINISTRATION OF SULPHONE†

Animal		Concentration of drug—mg./100 ml. or g.																				
		Liver	Lung	Spleen	Kidney	Intestine	Heart muscle	Fat	Urine	Brain	CSF	Intestinal lymph node	Skin	Blood	Udder								R. Supramammary lymph node
															Fluid				Tissues				
															RF	RH	LH	LF*	RF	RH	LH	LF*	
Cow 229		1·4	0·3	0·1	1·7	0·8	0·1	1·4	—	0·2	—	0·5	0·4	2·4	3·6	2·8	340	0·8	1·5	0·9	16	0·6	0·9
Cow 231		1·4	—	0·4	1·6	0·8	0·1	1·0	22	0·1	—	0·8	0·75	1·8	280	1·3	440	1·6	14	4·0	13	0·5	0·2
	Average	1·4	—	0·25	1·65	0·8	0·1	1·2	—	0·15	—	0·65	0·57	2·1	141·8	2·05	390	1·2	7·75	2·45	14·5	0·55	0·55
Calf 241		1·12	1·4	0·78	1·56	1·02	1·56	0·62	12	0·44	0·62	—	0·96	1·6									
Sheep 248		4·18	1·72	2·5	3·8	1·68	2·4	0·6	7	1·5	1·0	1·7	1·7	1·6									
Sheep 249		3·2	1·2	1·72	2·43	0·7	1·56	0·44	18	0·9	0·9	1·34	1·2	1·6									
	Average	3·69	1·46	2·11	3·11	1·19	1·98	0·52	12·5	1·2	0·95	1·52	1·45	1·6									

† Animals were given approx. 100 mg. of sulphone per kg. See text for details.

* This quarter was not injected.

The findings were statistically examined by Dr. O. L. Davies and the results are shown in Table IV. It will be seen that the concentrations in the brain and liver of the small mice were significantly higher than in large mice. Further, there was no significant difference between the concentrations in the blood and brain of small mice but in large mice the concentration in the brain was significantly lower than in the blood.

TABLE IV.

BLOOD AND TISSUE CONCENTRATIONS OF SULPHONE IN MICE GIVEN 0·1% IN THE FOOD FOR FOUR DAYS

	Blood	*Liver*	Brain
Large mice	1·35	2·45	0·68
95% limits of error ..	1·19—1·54	2·15—2·79	0·59—0·77
Small mice	1·71	3·63	1·59
95% limits of error ..	1·45—2·02	3·08—4·28	1·35—1·88

Nineteen large mice and twelve small mice were examined. The figures shown are logarithmic averages: the standard deviation per animal was 33 per cent. This standard deviation is appropriate for comparing large mice with small mice. A different standard deviation is required for comparing the concentration in the blood and brain of large and small mice; the standard deviation of the ratio of blood to brain concentration is 46 per cent per animal, from which it follows that in small mice there is statistically no significant difference between blood and brain concentration, whereas in large animals the difference is statistically highly significant.

DISCUSSION

Only a few observations have been made on the distribution of sulphonamides. The results of Gage, Martin, Rose, Spinks and Tuey (1947) support the view that concentrations in the tissues are no higher than in the blood, but there is ample evidence in this paper that in most species concentrations of sulphone are higher in the liver than in the blood. The results in the bovine are in direct contrast in that even in the liver the concentration of sulphone is lower than in the blood. If one assumes that sulphone is more toxic when in the tissues than in the bloodstream these results would provide a simple explanation of the low toxicity of sulphone for cattle. The big difference between the concentrations in the brain of cattle and sheep is surprising, and probably explains the lack of nervous symptoms in cattle and their frequence in sheep. All the differences in toxicity cannot, however, be explained simply on the basis of distribution: thus the bovine seldom or never develops methaemoglobinaemia, whereas the sheep does a little, and other animals to a much greater extent (Francis, 1949). The concentration of sulphone was higher in the brain of one calf than in two cows, although it was still relatively low. The higher concentration of sulphone in the brain of young than old mice would appear to explain the greater liability of young mice to develop nervous symptoms.

SUMMARY

Concentrations of sulphone were higher in the liver than in the blood of the mouse, pig, dog and sheep, but not of the bovine. The concentrations were not consistently higher in the skin than in the blood of mice, pigs, dogs and sheep. In the bovine they were lower. The concentrations in other tissues differed little from those in the blood. The lower concentrations in the tissues of the bovine may partly explain the lower toxicity of sulphone in this species.

Concentrations in the brain of sheep were only slightly lower than in the blood, whereas in the adult bovine they were much lower. This may explain the much lower tendency of cattle than sheep to develop nervous symptoms.

Concentrations of sulphone in the brain of young mice were relatively and absolutely greater than in old mice, which again may explain the greater liability of young than old mice to develop nervous symptoms.

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I am indebted to Dr. O. L. Davies for the statistical examination and to Mr. W. Russell for technical assistance.

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PHAGOCYTOSIS OF INTESTINAL BACTERIA IN THE APPENDIX OF NORMAL RABBITS

By

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INTRODUCTION

Many years ago, Bizzozero (1885), Ribbert (1885) and Ruffer (1890) observed that particulate matter such as bacteria may be seen in the lymphoid structures of rabbit's appendix and in recent years this observation has been confirmed (Baker and Enticknap, 1943; Digby, 1913, 1950). The process is of interest for three reasons: (1) it suggests a possible mechanism by which the host animal may utilise products synthesised by the bacteria of the gut; (2) it may constitute a protective mechanism against infection from the same source; and (3) it may thus have a bearing on the incidence of appendicitis in man and the few animals that possess an appendix. Suppurative appendicitis, if it occurs spontaneously in the rabbit must be extremely rare (Blount, 1945) and this high natural resistance may be due to the presence of a cellular protective mechanism absent from the human appendix.

Further investigation of these problems first requires an adequate description of the histological picture of this invasion and phagocytosis, but we have been unable to find such a description. Crabb and Kelsall (1940) have described the lymphoepithelium and discussed its histogenesis but do not mention the presence of bacteria in it. Jolly (1923) gives a good general account of the lymphoid structures present, and lymphopoiesis in this situation has been studied by Latta (1921) and Ehrich (1929).

In view of the fact that the entire phagocytic process in the rabbit appendix appears to have escaped the attention of many competent observers, it can scarcely be over-emphasised that the demonstration of the ingested bacteria is unconditionally dependent upon the use of appropriate methods.

This article gives an account of the phagocytic process in the rabbit appendix and emphasises features of the general histology which are necessary for its proper evaluation—for example, the lymphopoietic process and the migration and subsequent fate of the lymphocytes. The number of rabbits used in the present and similar studies is now over two hundred, and the histology is found to be remarkably uniform.

MATERIALS AND METHODS

Most of our specimens have been fixed with Bouin's fluid but 10 per cent formol saline has also been used and Carnoy's fixative for the study of migrating lymphocytes.

Bacteria were best shown by Gram's method or a modification using acetone as a decolourising agent, but for the demonstration of cytological detail, Giemsa preparations differentiated in colophonium alcohol were more satisfactory. The iodophile reaction of the bacteria was studied in sections stained with iron-hematoxylin, strongly differentiated, and mounted in 1 per cent iodine in 50 per cent aqueous glycerin. For smears the panoptic method of Pappenheim (1912) was used.

Phase-contrast microscopy was employed for the observations on living cells at magnifications of × 480 in air and × 1100 with an oil-immersion objective. Slide preparations were contained in a closed warm stage thermostatically controlled at 38°C during the actual observations. They were made from the appendices removed after the rabbits had had their necks broken. The organ was gently opened and scraped or washed so that it was free of its usual contents, and impression preparations were made from the mucosal surface. "Migration preparations" were made by allowing a coverglass to rest on the mucosa for 5 to 15 minutes in a sealed dish at 38°C and then transferring it to a slide to which it was sealed with vaseline.

Micro-incineration was carried out in a muffle furnace at 500°C and several standard histo-chemical tests were employed.

Appendicectomies were performed under general anesthesia with ether.

GENERAL HISTOLOGY

The structure of the wall of the appendix is that usual in the mammalian gut, but greatly modified by hypertrophy of the submucous layer (fig. 1). The two muscular laminae together do not make up one tenth of the thickness of the wall, the serosa is inconspicuous and the mucosa itself is not more than several cells thick. In ten rabbits the organ was found to be from 7 to 10 cm. long and the average thickness of the wall was 3·2mm. so it is clear that the components of the submucosa are present in very large amounts. Further, in three rabbits, the average weight of the appendix was 6·9g. and that of the spleen and mesenteric lymphatic mass combined was only 4·0g. so it seems possible that about half of the lymphatic tissue in the rabbit is in its appendix.

The mucosa.

This is a mucus-secreting intestinal epithelium modified by the presence of papillae of lymphatic tissue in the sub-mucosa. Each of these projects into the depth of a crypt of Lieberkuhn so that the latter becomes cup-shaped, 'Le Chalice' of Jolly (1923) (fig. 2). Three mucosal regions can therefore be recognised each of which has its own characteristics.

The mucosa of the papilla. There is extensive infiltration of this epithelium by intact medium-sized lymphocytes, except at the tip,

which largely escapes invasion. Goblet cells are entirely absent so that here it is covered by unmodified columnar epithelium.

The mucosa of the main cavity. This is regular, few goblet cells are present and lymphoid infiltration is restricted.

The mucosa of the crypts. Goblet cells are very numerous but cease abruptly at the base of the crypt where the epithelium becomes continuous with that of the papilla. (fig. 2). Extensive infiltration with small lymphocytes is seen but as they do not form nests there is little deformation of the epithelium.

The connective tissues and propria of the crypts

There is a continuous open network of collagenous fibres which surrounds and supports the nodules, becoming continuous with a typical reticulin mesh. Close to the fibres are primitive mesenchymal cells and fibroblastic activity is seen. There are relatively few lymphocytes, macrophages or mesenchymal cells in this region but moderate numbers of plasma cells and some coarsely granular eosinophiles are present.

The submucosa

In transverse section this is seen to consist of thirty to forty large nodules distributed radially (fig. 1). These present three broad zones (see figure), the papilla, separated from the voluminous basal zone—the panse or 'renflement' of Jolly—by a dense band of small lymphocytes. This middle zone is continuous laterally with those of adjacent nodules as the 'ailerons' of the same author. The lymphatic channels are well developed and resemble those found in man (Seng, 1932).

THE CYTOLOGY OF THE SUBMUCOSAL LYMPHOID NODULE

This comprises a reticular syncytium of mesenchymal cells, macrophages, lymphocytes in all stages of development and plasma cells. The cells correspond closely to the descriptions given for human material by Robb-Smith (1938). The macrophages which alone require further description are distributed throughout the entire nodule but attain their greatest density in the papilla. In the basal zone they occur in aggregates related to the peripheral lymph spaces. Mitoses have never been seen in them and wherever macrophages occur a few primitive mesenchymal cells can also be seen.

THE PHAGOCYTIC ACTIVITY OF THE NODULE

Such macrophages are active phagocytically and invariably contain a variety of inclusions and ingested bacteria (fig. 4). Basophil inclusions are easily identified as normal or pyknotic nuclei, probably of lymphocytes, but the acidophile inclusions show no organised structure by phase-contrast microscopy or other methods. The peripheral aggregates contain large amounts of refractile lipoid and chromophobe materials, together with some carbon particles

and materials that give a positive reaction for iron. A combination of micro-incineration and the application of hydro-fluoric acid was found necessary completely to remove these deposits so they must also contain some compounds of silicon. They are therefore probably exogenous in origin.

Single micro-organisms may often be observed within clearly defined vacuoles (fig. 4) and undergoing digestive changes, (figs. 5 and 6) but more frequently the bacteria are aggregated in large clumps (figs. 7 and 8). This clumping is not seen among the numerous bacteria lying outside the cells (fig. 9). Bacteria have not been seen in the propria of the crypts.

There can be no reasonable doubt, that the ingested organisms have their origin in the lumen of the appendix since morphologically identical organisms can be seen in the lumen and extending into the crypts and those in all three situations can be stained with iodine. By this means many of the phagocytosed rods are stained blue while others, in various stages of digestion, are stained a more reddish colour or remain colourless.

The process is however selective. The microbial population of the appendix is similar to that of the caecum of the rabbit which includes a wide variety of micro-organisms (Baker and Martin, 1937), and of these only rod forms are present in the papilla and are ingested by the macrophages.

The consistency of the contents of the lumen is that of a thick suspension which can therefore readily pass into the crypts. Having done so the microbial mass adheres to the surface of the papilla as a nearly continuous film, whereas it is merely pressed into loose contact with walls of the crypt (fig. 10).

Despite the presence of this extensive bacterial invasion, to which we know of no parallel in any other normal tissue, only inconsiderable numbers of granulocytes have been seen in the numerous sections examined. This is perhaps the most remarkable feature of such a process occurring in mammalian tissue.

THE LYMPHOPOIETIC ACTIVITY OF THE NODULES

This takes place mainly in the basal zone of the nodules. (fig. 11). Although the general appearance here suggests a follicular structure the division into nodules is probably a result of the disposition of the trabeculae. Physiologically the nodules are more closely related to diffuse lymphoid tissue than to the reactive centres seen in the human appendix and the mitoses seen probably represent significant lymphopoiesis. The special collecting venules of Ehrich (1929) can also be seen here. We have been unable, however, to obtain positive results indicating that this process has any functional significance for the rabbit. No significant differences have been observed in the general health, growth rate, microscopic features of alimentary contents or blood count of five appendicectomised young adult

animals when compared with litter-mate controls and kept on a green diet.

THE MIGRATION OF THE LYMPHOCYTES

In sections, nests containing two to ten lymphocytes are seen in the papillary mucosa. (Fig. 12). The border of the nest is formed of a hyaline acidophile substance and within this roughly spherical casing can be distinguished the separate cytoplasms of the individual lymphocytes surrounded by clear unstained material. (Fig. 13). Some of these have irregular outlines highly suggestive of pseudopodial formation (Williamson, 1950). The epithelium is greatly deformed by the presence of these nests and appearances suggesting the coalescence of contiguous nests have been seen.

Crabb and Kelsall (1940) state that the majority of the lymphocytes in this situation have been engulfed by macrophages and suggest that this is the method of transport. In the present investigation no definite evidence of this process could be found and if phagocytosis really occurred here it would almost certainly involve bacteria as well. In fact, although single organisms may be found, neither clumps of bacteria nor any lytic cellular changes were seen. It is believed, therefore, that macrophage activity plays no significant part in the migration across the epithelium in either direction.

Experimental observations

Observations on living cells make it clear that these lymphocytes do, in fact, actively extrude pseudopodia as was observed by Ebert, Sanders and Florey (1940) for those in the blood stream. Only very slow translation has been observed and motility is reduced or absent if a suspending fluid such as Ringer's is used.

In the migration preparations numerous cells were seen, all those recognisable being medium-sized and large lymphocytes. Many of the cells are in compact groups as they appear in the nests in the sections. Neither intra-cellular lymphocytes nor any macrophages have been seen in preparations incubated for less than fifteen minutes. After this time autolysis frees epithelial cells and the picture is much confused. These observations have been checked by fixation and subsequent staining.

No nuclear chromatin changes were observed during prolonged observation of the living cells, in keeping with the observed rarity of mitosis in sections of this region.

If it be assumed that conditions in the fifteen minutes immediately after excision are representative of those obtaining *in vivo*, an estimate can be made of the magnitude of the process. Something of the order of 1 c.mm. of lymphocyte mass, estimated by direct measurement of fluid volume and a visual estimate of ratio of cells to fluid, collects on the coverslip in this period. From this it follows

that the volume of lymphoid tissue in this organ (approximately 5 mls. estimated from tissue weight and a visual appreciation of relative amounts of the tissues seen in sections) is passed into the gut every few days, a period comparable to the assumed life of lymphocytes (Yoffey, 1933). On these assumptions it may well be that only an insignificant number of lymphocytes here formed reaches the blood stream, although the possibility that the migration may take place in both directions cannot be ignored. Indeed lymphocytes in smears from the lumen and in this region in sections, are often quite intact.

DISCUSSION

The structure of the rabbit's appendix is such as to make it seem reasonable that its function is not merely passively to permit, but actively to promote bacterial invasion of the wall from the lumen. For, by the secretion of mucus, the bacteria-rich contents of the organ will be pushed away from the walls of the crypt (Fig. 10) into close proximity with the surface of the papilla. In consequence invasion will be canalised so that the invading micro-organisms are brought immediately into the vicinity of the numerous macrophages by which, subsequently, they are taken up and digested.

Successful invasion of the papilla, moreover, depends upon the ability of bacteria to penetrate the mucosa, since macrophages appear to play no part. This may be made possible by a deformation of the epithelium consequent upon the formation of lymphocytic nests, as bacteria are found in large numbers only in the parts of the submucosa over which the epithelium is so deformed. Since only rod forms are seen in the submucosa, and the majority of strains isolated from that site were found to be motile (Enticknap and Baker, 1946) it is probable that motility is an essential characteristic of the tissue flora and that local histogenous factors do not adequately explain their presence.

In this interpretation the outward migration of lymphocytes towards and the inward migration of bacteria from the lumen are complementary features of a mechanism organised so as to facilitate the entrance and secure the destruction of the intestinal bacteria. It must, however, be admitted that actual migration of the lymphocytes into the lumen of the gut has not been proven by these experiments, for they do not reproduce natural conditions sufficiently closely. This does not, however, affect the main argument that the epithelial deformation produced by the presence of these nests facilitates the ingress of motile bacteria.

The existence of this mechanism clearly has bearings upon pathology (Ruffer, 1890), immunity (Digby, 1950) and nutrition (Baker and Enticknap, 1943) but its significance can only be determined by further experiment.

SUMMARY

A brief account of the histology of the appendix of the rabbit is given. The invasion of the lymphoid structures of the rabbit appendix by bacteria from the lumen and the subsequent ingestion and digestion of the invading micro-organisms by the macrophages is described. The invasion appears to be canalised by the secretion of mucus from the adjoining crypts of Lieberkuhn and the deformation of the mucosa covering the papilla by the presence of lymphocytes. The process may be a protective mechanism or a means to the utilisation of microbially synthesised vitamins by the host animal, and may provide an experimental method for studying appendicitis.

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LEGENDS TO ILLUSTRATIONS

Fig. 1. Section of whole thickness of wall of rabbit's appendix to show the high proportion of the submucosa relative to the other layers and the differentiation of it into three zones. (H & E, × 23)

Fig. 2. Showing the situation of the lymphoid papilla in relation to Lieberkuhn's crypt. Goblet cells are absent from the papillary mucosa and are numerous in the walls of the crypt. (Leishman, × 60)

Fig. 3. Typical structure of macrophages (Gram-Weigert, × 75).

Fig. 4. Impression preparation of lymphoid tissue to show macrophages with vacuoles and ingested material (Panoptic Giemsa, × 1400)

Fig. 5. Clumped bacteria before digestion (Gram-Weigert, × 1800)

Fig. 6. A similar group in an advanced stage of digestion (ditto)

Fig. 7. Group of bacteria showing appearance suggesting agglutination (Gram-Weigert, × 750)

Fig. 8. Clumps of bacteria in macrophages (ditto)

Fig. 9. Bacteria in interstices of lymphoid tissue (ditto)

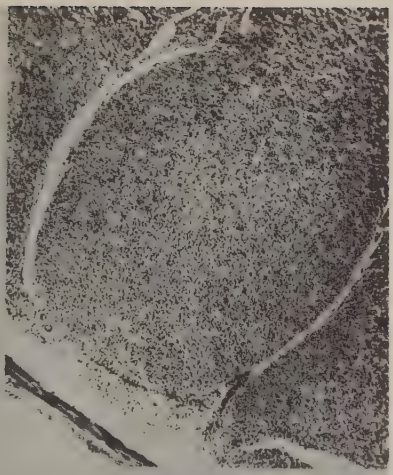
Fig. 10. Effect of secretion of mucus on disposition of lumen contents (H & E, × 350)

Fig. 11. Base of lymphoid nodule (H & E, × 80)

Fig. 12. Mucosa of papilla and crypts showing presence of nests in former only (H & E, × 620)

Fig. 13. Mucosa of papilla showing lymphocytes in nest (Iron hematoxylin, × 1600)

Fig. 1.



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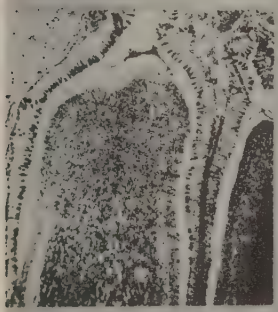

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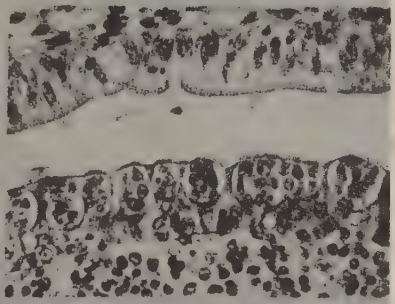

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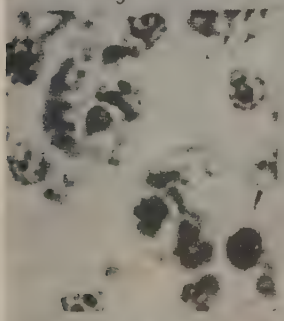

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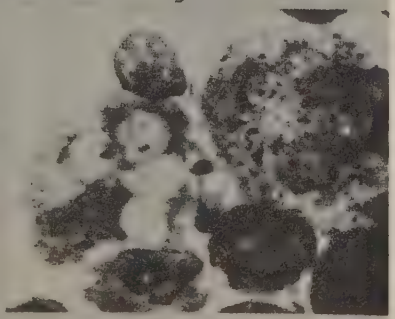

Fig. 4.

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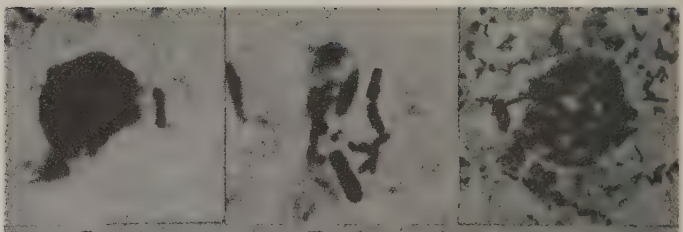

Fig. 5. Fig. 6. Fig. 7.

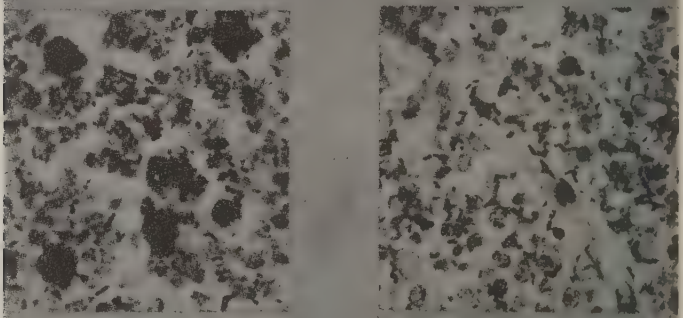

Fig. 8. Fig. 9.

Fig. 10. Fig. 13.

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THE EFFECT OF CORTISONE ON THE CONCENTRATION OF *SALMONELLA PARATYPHI A* AGGLUTININS IN THE SERUM OF IMMUNISED RABBITS

By

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INTRODUCTION

Divergent results have been obtained by workers investigating the effect of adrenal steroids on the concentration of circulating antibody in rabbits. Chase, White and Dougherty (1946), using serial dilution methods of determining antibody concentrations, found that the administration of adrenocortical extracts was followed by an increase. Other workers, employing similar methods of antibody estimation, failed to demonstrate any effect after the administration of cortisone acetate (Katzin and Goldman, 1950), or other cortical preparations (Mitchell, Fulton, Talbot and Kimball, 1951). Depression of antibody concentration after treatment with cortisone was reported by Bjørneboe, Fischel, and Stoerk (1951), who used microchemical methods of determining antibody nitrogen, and by Germuth and Ottinger (1950), using spectrophotometric methods.

The present paper records an experiment in which rabbits were treated with cortisone acetate during the process of immunisation with a killed suspension of *Salm. paratyphi A*, and the "H" agglutinin titres of their sera compared with those of littermates which had been immunised but not treated with cortisone.

MATERIALS AND METHODS

A suspension of *Salm. paratyphi A* was prepared by growing a freeze-dried culture in nutrient broth at 37° C., subculturing on to a series of agar slopes overnight, and washing off with 0·1 per cent formaldehyde in normal saline. The suspension was tested for sterility and standardised to a density of 1,000 million organisms per ml.; it was used both for immunisation and for antibody estimation.

Thirteen litters of rabbits were used at different times; their average weights were between 1·3 and 2·4 kg. They were fed on the pelleted diet of Bruce and Parkes (1947), supplemented with unlimited raw cabbage, and with water during hot weather. All were given an injection of 10 mg. of ascorbic acid once weekly, and were weighed twice weekly, throughout the experimental period.

Four animals were used from each of the first six litters. Each of the four was subjected to a different regime and marked accordingly for identification purposes as follows:

A—Untreated Control. This animal was given no special treatment apart from that already mentioned.

B—Immunised. This animal received an injection of the *Salm. paratyphi A* suspension into an ear vein twice or thrice weekly. The first injection was of 0·1 ml.; the dose was raised by increases of 0·1 ml. to 0·4 ml., at which level it was maintained. Immunisation continued for three weeks.

C—Cortisone-treated. This animal was given subcutaneous injections of cortisone acetate (Merck), 5 mg. per kg. body weight per day, on five or six days a week for three weeks.

D—Immunised and Cortisone-treated. This animal was immunised on the same days and with the same doses as animal B, and was given cortisone on the same days and in the same doses as animal C.

In the last seven litters the "cortisone control" (C) was omitted, and three rabbits in each litter were subjected to the A, B, and D regimes respectively. In litter no 5, animal B died of an intercurrent infection shortly after the start of the treatment. Animal A of this litter was then subjected to the B regime, and was regarded thenceforth as a B.

Before and immediately after the three weeks' treatment a sample of blood was removed from the ear veins of each rabbit. The antibody nitrogen content of the first serum specimens of litters 1 and 2 was determined by a micro-Kjeldahl method. In all the remaining serum specimens, agglutinin titres were determined by a serial dilution method; the reasons for this change are given in the discussion. A titration in doubling dilutions sufficed for the serum specimens taken at the start of the regimes, and for the final specimens of the A and C series. In the case of the final specimens of the B and D series a more accurate estimation was then performed over a narrow range of serial dilutions above the previous endpoint. All agglutination tubes were incubated at 37° C. for 15-18 hours.

RESULTS

The initial antibody concentrations were either negligible (litters 1 and 2), or gave titres which did not exceed 1/40 (litters 3 to 13).

In the A and C series (18 animals) the final agglutinin titre did not exceed 1/40 in 14 of the animals. In 3 of the remainder (1A, 3C, 4C) the final titre was 1/80. In one animal (9A) it was 1/640. No reason for this could be found as the animal remained apparently healthy throughout. However, this titre is low as compared with that of its littermates in the B and D series. Animal 4D developed an intercurrent infection and had to be killed three days before the scheduled end of its regime. The final titre recorded is that at death. The serum of animal 7D clotted after the preliminary (doubling dilution) estimation had been performed, and the antibody titration could not be completed.

Table 1 gives the reciprocals of the final titres of the animals of series B and D. The difference between the reciprocal of the titre in the endpoint tube and that in the next tube in either direction is also shown. All figures are in thousands. When the variations in the two sets of figures are tested statistically, and omitting litter 4 from consideration, the difference between the series is found to be significant and not due to chance variation or random errors inherent in the methods used. The results indicate that the serum of the cortisone-treated animals (series D) contained less detectable antibody than that of the untreated animals (series B).

TABLE 1.

FINAL ANTIBODY TITRES
(reciprocals, 000s omitted)

Litter No.	*Vaccine series (B)*	*Vaccine and Cortisone series (D)*
1.	120 ± 8	16 ± 4
2.	18 ± 4	36 ± 8
3.	40 ± 4	20 ± 4
4.	64 ± 8	1 (at death)
5.	200 ± 20	20 ± 4
6.	120 ± 10	14 ± 2
7.	126 ± 7	26 (doubling dilutions)
8.	56 ± 4	24 ± 2
9.	47 ± 4	19 ± 2
10.	64 ± 8	72 ± 8
11.	96 ± 16	18 ± 2
12.	120 ± 20	66 ± 6
13.	32 ± 4	33 ± 10

The significance of difference between the two series is $p < 0.001$

In 7 out of the 13 animals of series D the serum was markedly lipaemic. Some of the livers of the D animals were examined histologically; in nearly all of these the hepatic cells were distended with glycogen. As shown in Table 2, the D animals lost more weight or gained markedly less than their littermates; it was obvious during the course of the experiment that this was due largely to muscular atrophy. These findings suggested that increased protein catabolism and fat metabolism had taken place.

TABLE 2.

RATIOS OF FINAL TO INITIAL WEIGHTS (× 100)

Litter	*Series: Control (A)*	*Vaccine (B)*	*Cortisone (C)*	*Vaccine and Cortisone (D)*
1.	103	98	96	85
2.	115	110	89	78
3.	167	139	131	115
4.	121	115	106	87 (at death)
5.	127	—	106	93
6.	123	118	94	94
7.	91	97		84
8.	124	131		94
9.	138	133		120
10.	111	105		100
11.	129	128		113
12.	135	129		108
13.	125	122		106

DISCUSSION

The original intention was to perform all antibody estimations by micro-Kjeldahl determinations of antibody nitrogen (Kabat and Mayer, 1949), but the experimental error of the method proved so great as to mask any possible differences in antibody concentration in the unimmunised animals. The method appeared to possess no advantages over the serial agglutination method when dealing with a single antigen-antibody complex, and was considerably more laborious. It may be said of the latter method that it does not give a true measure of antibody concentrations, and that some of the large differences of titre found between cortisone-treated and untreated animals (e.g. between 5D and 5B) probably do not in fact represent the degree of difference in antibody concentrations. There are grounds for such a criticism, but it may also be levelled against the method of microchemical determination of total antibody nitrogen. It is clear, however, that in the present experiment the depression of circulating agglutinin in the cortisone-treated animals was sufficiently great to be detected by a serial agglutination technique.

Among possible interpretations of this finding is that of Bjørneboe *et. al.* (1951), who considered that the antibody depression was related to the atrophy of lymphoid tissue and to the diminution in the numbers of certain types of mononuclear cells which they observed in their cortisone-treated animals. Recent work, however, has indicated that antibody production is a property possessed by many other types of animal tissue (Oakley, Batty and Warrack, 1951). An alternative explanation, supported by our findings, is that the antibody depression is a manifestation of increased protein catabolism. A third possibility, suggested by Clearkin (1952) is that circulating cortisone alters the physico-chemical properties of serum in such a way as to mask antigen-antibody reactions.

The striking effect of cortisone on the body weight of the treated animals is worthy of note, and appears to support the second of the above three possibilities.

SUMMARY

Less agglutinin could be detected in the serum of immunised and cortisone-treated rabbits than in that of their immunised but untreated littermates.

Cortisone-treated rabbits gained less weight or lost more than their untreated littermates subjected to otherwise identical regimes.

It is suggested that these effects were due to increased protein catabolism.

ACKNOWLEDGMENTS

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OBSERVATIONS ON TUBERCULOSIS IN THE FERRET (*MUSTELA FURO* L.)

By

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INTRODUCTION

Knowledge of the characteristics of tuberculosis in the ferret (*Mustela furo* L.) and of the measures by which the infection can be prevented is particularly important to those who use this animal in the laboratory. The ferret is highly susceptible to infection by certain types of *Mycobacterium tuberculosis* and the disease may occur in a form which readily escapes detection, so that it may be transmitted unwittingly by inoculation or other experimental procedures.

Little is known of the incidence of tuberculosis in the ferret as adequate post-mortem studies have not been numerous. The scanty literature on the subject can be reviewed briefly. The disease received only passing mention in Lagoutte's (1935) monograph and in the account by Hughes (1947) of the management of the ferret as a laboratory animal. Dunkin, Laidlaw and Griffith (1929) described three cases of spontaneous generalised tuberculosis in ferrets and isolated a bovine tubercle bacillus from one of them. In a small series of experimental studies these workers found that the ferret was highly susceptible to infection by bovine tubercle bacilli introduced subcutaneously or by mouth, whereas human tubercle bacilli produced only indolent local lesions. As in the spontaneously infected animals, the lesions which developed in experimental infections with bovine bacilli contained very large numbers of organisms; only a few organisms were found in the lesions in animals infected with human bacilli. These observations were confirmed by Goret and Mariette (1939), who in addition investigated the pathogenicity of avian tubercle bacilli to the ferret and described their findings as similar to those obtained with the human type of organism.

We have found no published reference to spontaneous infection of the ferret with human or avian strains of tubercle bacillus, and Wilson and Miles (1946) mentioned only the bovine bacillus in referring to tuberculosis in this species. MacIntyre (1951), however, has told us that in his experience of tuberculosis amongst many thousands of ferrets bovine strains have been very much less frequent than avian strains; more than 60 per cent. of those ferrets which he obtained from dealers were tuberculous. In contrast to the fatal generalised disease caused by bovine bacilli, avian-bacillus infection seems to do the ferret little harm, apparently causing a chronic Yersin type of disease, with splenomegaly but no macroscopical tubercles. MacIntyre (1951) stated that avian bacilli caused the infection in the ferrets mentioned by Dalling and O'Brien (1935). Spanedda (1941) isolated from a spontaneously infected ferret an organism which he considered to be a new type, *Mycobacterium*

mustelae, intermediate between the "paratubercle bacilli" and true tubercle bacilli; this organism thrived in broth and other simple media as well as on Petragnani's medium, and was pathogenic to the guinea-pig but not to the rabbit or fowl.

Other members of the genus Mustelida, to which the ferret belongs, include the polecat (*Putorius putorius* L.), stoat (*Mustela erminea*), weasel (*Mustela vulgaris*) and mink (*Mustela vison*). Of these, the mink is the only species in which tuberculosis has to our knowledge been described. Saenz, Truche and Costil (1934) described one case, due to infection with a bovine tubercle bacillus, and Schoop (1940) defined two clinical forms of the disease, a rapidly fatal variety characterised by great ascites and little or no wasting, and a chronic emaciating form with severe pulmonary involvement. Both Schoop (1951) and Sprehn (1951) have isolated only bovine bacilli from tuberculous minks. Sprehn (1951) has told us that tuberculosis is the commonest infection on German mink-farms, accounting for 12·2 per cent of deaths in his necropsy material in 1950, and that the incidence of the disease is greatest in those districts where uncooked offal is fed to the animals. In contrast to the experience of the German workers, Gorham (1951) has told us that tuberculosis is rare in minks in North America, where the disease has been largely eliminated in cattle, which are a source of meat for minks.

Tuberculosis in the ferret is preventable. Boiling all milk and substituting other forms of flesh by horse-meat and fish prevent food-borne infection; it is also essential to exclude from the laboratory colony any ferrets which are not obtained from completely trustworthy sources.

Since 1947, over 700 ferrets have passed through the colony of these animals which is maintained for endocrinological research in the anatomy department of the University of Birmingham. Just over 600 of these animals have come to necropsy and only one case of spontaneous tuberculosis has been recognised. About half of the animals were obtained from commercial sources; the others were bred in the colony or in other institutes where strict precautions to prevent tuberculosis were enforced.

REPORT OF CASE*

History. The animal (T.F. 430) was a mature female albino ferret, obtained from an institute where tuberculosis was thought to have been eradicated from the ferret colony. It appeared to be in perfect condition when we received it; its weight was 710 g., which is well within the range of the normal weight of the fully-grown female (450 to 1,000 g.). On the next day the animal was noticed to walk with difficulty. One day later all its limbs were weak; the hind limbs were widely splayed, so that the belly dragged on the ground. During the next three days the extent and severity of the paralysis increased; the animal was therefore killed with chloroform vapour.

* *An abstract of some of our findings has been published elsewhere (Iland, Symmers & Thomson, 1951).*

Post-mortem examination. Necropsy was performed immediately after death. The abdominal cavity contained about 10 ml. of turbid yellow fluid, and the parietal peritoneum was closely studded with firm greyish nodules, 0·05 to 0·3 cm. in diameter. The blood vessels in the omenta and mesenteries were surrounded by sleeves of white tissue, 0·1 to 0·2 cm. wide. The stomach and intestines were greatly indurated by coalescent plaques of similar tissue in the subserosa and to a smaller extent beneath the mucosa. The liver (44 g.) and spleen (14 g.) were little, if at all, enlarged. The cut surface of the liver was normal; the spleen contained many ill-defined grey nodules, 0·1 to 0·25 cm. in diameter. The abdominal lymph nodes were enlarged and their substance was almost entirely replaced by firm, opaque, whitish tissue which was outlined by a hair-fine, ochre-coloured rim. The ureters, urinary bladder and reproductive organs were partly buried in similar whitish tissue. The kidneys and adrenal glands were normal.

Each pleural sac contained about 4 ml. of turbid yellow fluid. The parietal pleura was normal. Firm yellowish nodules, 0·15 to 0·3 cm. in diameter, were scattered through the lungs. A caseous mass occupied the site of the upper retrosternal lymph nodes. The pericardial sac was normal. Sleeves of firm whitish tissue surrounded the larger coronary vessels. Barely visible vegetations were scattered sparsely on the surface of the aortic valve cusps and on the intima of the aorta. The respiratory passages, pharynx, oesophagus, cervical lymph nodes and thyroid gland were normal.

There were no apparent lesions in the pituitary gland, meninges, brain or spinal cord. The adductor muscles of the thighs were yellow and soft; the rest of the musculature was normal. The bone marrow was hyperaemic and studded with minute grey nodules. The joints were normal.

Microscopical examination. The tissues were fixed in formol-saline solution (4 per cent. formaldehyde). The most striking histological features were the enormous numbers of acid-fast bacilli in the tissues and the histiocytosis and necrosis associated with their presence. Epithelioid metamorphosis of the histiocytes was nowhere conspicuous; a few cells had more than one nucleus but no Langhans cells were seen. The necrosis was predominantly coagulative, with preservation of the general architecture of the tissues; caseation was seen particularly in the walls of the hollow organs, although there was also a widespread caseous perivascular lymphangitis. Many of the blood vessels remained intact in the midst of extensive necrotic foci and were surrounded by a narrow zone of surviving tissue infiltrated by histiocytes and lymphocytes (Fig. 1). Acid-fast bacilli were so numerous in the necrotic tissue, particularly towards the periphery of the lesions, that Ziehl-Neelsen preparations appeared red to the unaided eye (Fig. 2). Many degenerated histiocytes packed with bacilli remained recognisable in areas of coagulative necrosis.

The normal structure of the enlarged lymph nodes survived only in small areas around the hilus; elsewhere it was replaced by necrotic tissue, and the ochre-coloured rim noted macroscopically coincided precisely with the greatest concentration of bacilli. There was considerable fibroblastic proliferation and fibrosis in the capsule of the lymph nodes, although fibrosis was slight or absent in other lesions. Much of the normal tissue of the spleen and bone marrow was replaced by a diffuse accumulation of histiocytes, with innumerable small areas of caseation in which bacilli were particularly abundant. There was no necrosis in the liver; scattered aggregates of histiocytes and lymphocytes were present in the portal tracts, and there was a nodular hyperplasia of Küpffer cells, which also contained bacilli. Small caseous foci were present in the gastrointestinal mucosa and there were many bacillus-laden histiocytes throughout the mucosa; there was no ulceration.

The lesions in the lungs differed from those elsewhere. The alveoli within the nodules were packed with foamy phagocytes filled with sudanophile fat and with minute needles of birefringent lipid which showed little or no sudanophilia; these lipophages seldom contained bacilli. An occasional large acicular crystal of non-sudanophile, birefringent lipid was contained in a typical multinucleated foreign-body giant cell. The alveolar septa around the nodules were infiltrated by bacillus-laden histiocytes; as elsewhere, only a minute amount of fat was demonstrable in these histiocytes. The pulmonary nodules were the only macroscopical lesions which proved not to be necrotic.

The vegetations on the aortic valve and on the intima of the aorta consisted of a fibrinous matrix in which there were large aggregates of histiocytes packed with organisms. Some were completely covered by endothelium.

Histiocytes containing a few or many bacilli were scattered in small numbers throughout all the tissues except the substance of the brain and spinal cord. They were found between fat cells in adipose tissue, in the walls of blood vessels and beneath the endocardium, in the choroid plexuses, meninges and retinae, and sometimes in glomeruli and between renal tubules. Many mesothelial cells, particularly of the peritoneum, were swollen and contained a few bacilli (Fig. 4). Bacilli were present in some ependymal cells and in some cells lining the adrenal and pituitary sinusoids. They were seen in large mononuclears in the blood and in similar cells, apparently macrophages, in lymphatics (Fig. 4). Granular leucocytes were scanty in the tissues.

The only abnormality in the central nervous system was lysis of Nissl substance in some anterior horn cells at all the levels of the spinal cord examined. Sections of the cord and of peripheral nerves prepared by Marchi's method for demonstrating degenerating myelin, by Loyez's method for myelin and by Holmes's axon stain showed no abnormality. There was slight fatty vacuolation of skeletal

muscle fibres, some of which showed loss of striation and even severe hyaline swelling; these changes were most marked in the adductors of the thighs. There was no evidence of myositis.

The only other noteworthy histological observation was the presence of many extramedullary foci of haemopoiesis, particularly in the spleen, liver and mediastinal fatty tissue.

EXPERIMENTAL INFECTION OF A FERRET

A sexually mature male ferret (T.F.S.O.), 960 g. in weight, was infected by intramuscular injection with the organism isolated from T.F. 430. The inoculum was estimated to contain approximately 50,000 bacilli; it was prepared from the tissues of an infected rabbit, as described below. The ferret remained in seemingly perfect condition for six months after the inoculation, continuing to grow normally. At the end of this period it suddenly sickened and developed paralysis which within a few days spread from the adductor muscles of the thighs to affect all the limbs. Five days after the first sign of illness the animal was moribund and was killed with chloroform vapour.

Post-mortem examination. The cadaver weighed 1,280 g. (normal range in adult males: 900 to 1,500 g.). A fibrous nodule, 0·25 cm. in diameter, was found at the inoculation site in the adductor muscles of the right thigh. The adductors of both thighs were yellow and friable and there was patchy pallor of other muscles. The liver weighed 98 g. (normal maximum: 60 g.). The spleen filled most of the abdominal cavity and weighed 138 g., equivalent to almost 11 per cent. of the body-weight (the weight of the normal spleen does not exceed 20 g. and is usually considerably less). Both liver and spleen were firmer than normal and their pattern was patchily obscured by ill-defined, pinkish-grey areas which contrasted with the surrounding congestion. The abdominal lymph nodes were enlarged up to twice normal size but showed no other abnormality. The lungs contained a few firm, yellow nodules, about 0·25 cm. in diameter. The rest of the body was normal.

Microscopical examination. The tissues were fixed in formol-saline. The findings differed from those in T.F. 430 in the absence of necrosis, in the extraordinary degree of histiocytosis and in the considerably smaller number of organisms, all of which were intracellular. The diameter of the histiocytes ranged from 12 to 20μ; they contained little or no demonstrable fat; acid-fast bacilli were present in many, although there were seldom more than two or three in a cell.

Much of the parenchyma of the liver was replaced by a massive accumulation of large and small histiocytes (Fig. 5); large numbers of histiocytes were present throughout the portal tracts, in which small collections of lymphocytes and groups of haemopoietic cells were also present, as well as scattered neutrophile polymorpho-

nuclears. Nodular hyperplasia of Küpffer cells was seen in the areas where the parenchyma survived.

The splenomegaly was due to histiocytosis (Fig. 6). Small haemorrhages and small collections of lymphocytes or haemopoietic cells were present at the periphery of the histiocytic aggregates; occasional megakaryocytoid cells were seen and were indistinguishable from similar cells which occur normally in the spleen. The splenic follicles were small and widely spaced.

The appearances of the bone marrow were similar to those of the spleen; little haemopoietic tissue remained. The lymph nodes showed a relatively slight sinus and medullary histiocytosis; most of their enlargement was due to simple dilatation of the sinuses and lymphoid hyperplasia. Small foci of reticulo-endothelial-cell hyperplasia were seen in the adrenals and pituitary.

The pulmonary lesions were similar to those in T.F. 430; the alveoli contained very numerous multinucleated giant cells in which large lipoid crystals were present. No acid-fast bacilli were seen in the giant cells, although they were more numerous in the histiocytes within the interalveolar septa in the pulmonary nodules than anywhere else in the body.

Very scanty intracellular acid-fast bacilli were present in the fibrous scar at the inoculation site. Degenerative changes, similar to those in T.F. 430, were found in the muscles and anterior horn neurones. Apart from the presence of an occasional histiocyte containing one or two bacilli the other organs and tissues were normal.

BACTERIOLOGICAL STUDIES

The organism was isolated by inoculating a rabbit intravenously with 0·5 ml. of untreated ascitic fluid from T.F. 430. The rabbit died 22 days later with widespread tuberculosis which involved the lungs predominantly; smears of its viscera contained large numbers of acid-and-alcohol-fast bacilli, and histological sections confirmed the presence of typical acute tuberculosis. A saline suspension of ground-up lung was treated with sodium hydroxide, 4 per cent. by volume, for 30 minutes and then neutralised. Supernatant fluid decanted after sedimentation of the neutralised suspension was inoculated intramuscularly into the ferret T.F.S.O., guinea-pigs, rabbits and white rats. It was also planted out on Löwenstein-Jensen medium. The further cultural and pathogenicity investigations were carried out with the cultures obtained on this medium and with material from the infected animals.

Cultural characteristics. All the cultures were incubated at 37° C., unless the contrary is stated.

Dorset's egg medium and Löwenstein's medium: poor, rather confluent, almost pure white, dry granular growth; the colonies did not become heaped or nodular. Growth was not enhanced by incubation at 41° C. for one week.

Dubos's "Tween 80" medium: diffuse turbidity with some granules after one week; growth moderate in amount.

Growth of the organism was not helped by the addition of glycerol to the media. The results of cultivation on other media confirmed that the organism was dysgonic and non-pigmented. From its cultural characteristics it was provisionally classified as a bovine-type tubercle bacillus.

Morphology. In cultures and in the tissues the organism was a short, straight or slightly curved bacillus with smooth sides and rounded ends. Beading was apparent only after staining by Gram's method. The organisms did not form the usual twisted coils and clumps characteristic of most strains of tubercle bacillus, but spread evenly in smears, although sometimes with a tendency to lie side by side in a manner reminiscent of diphtheroid bacilli. Known bovine and human strains of tubercle bacillus, grown on the same batches of media as the organism under investigation, did not show this peculiarity of distribution in smears but formed coils in the usual way. The uncentrifuged exudate from the body cavities of T.F. 430 and its urine and faeces contained enormous numbers of the bacilli, which spread in the same way as the cultivated organism when smears were made.

Staining reactions. The organism was strongly acid-and-alcohol-fast in the tissues and on culture. When stained with auramine-O it fluoresced brilliantly in ultra-violet light. It stained less intensely with Giemsa's stain than is often the case with human leprosy bacilli in paraffin sections.

The results of Gram staining were unusual. In the serosal effusions of the spontaneously infected ferret, and in smears and sections (Fig. 3) of the tissues of both ferrets, the organisms were strongly Gram-positive when stained by the ordinary, rapid, routine Gram method at room temperature. Omission of the iodine step in the Gram routine resulted in rapid decolorisation of the organisms during subsequent treatment with alcohol or acetone, and they then stained with the counterstain. The bacilli stained rather less evenly with Gram's stain than with the Ziehl-Neelsen stain, and the former was sometimes concentrated in the beadings. It is noteworthy that in Gram preparations, whether smears or sections, no Gram-negative organisms were seen, except in foci of caseation where many of the extracellular bacilli stained feebly or not at all with Gram's stain. Similarly, all the organisms in Ziehl-Neelsen preparations were acid-and-alcohol-fast. Marked fields were photographed after Gram staining; when the preparations were decolorised with acid-alcohol and restained by the Ziehl-Neelsen method it was seen that the organisms which had been shown to be Gram-positive were also Ziehl-Neelsen-positive. The fact that the organisms were readily shown to be as strongly and consistently Gram-positive in simple smears of the exudates and tissues of the infected ferrets as they were in paraffin sections excludes the possibility that the reaction

with Gram's stain in the sections was an artifact due to the effects of the dehydrating and clearing agents used in preparing the tissues for imbedding in paraffin wax. This is an important point, as prolonged exposure of tubercle bacilli to these agents sometimes does indeed make the organisms readily stainable by the routine Gram's method, perhaps as a result of some effect on their lipoid constituents.

The staining reactions of the organism after one week's and four weeks' cultivation on glycerol-free and on glycerol-containing media were compared with those of standard National Type Collection strains of human, bovine and avian tubercle bacilli grown on the same batches of media for the same periods. The young cultures of the organism from the ferret were quite easily stained by the ordinary Gram method but did not differ greatly in this respect from the standard strains. The ferret strain lost its Gram-staining peculiarity in later cultures. No strain differences in acid-and-alcohol-fastness were observed.

Pathogenicity. Investigation of the pathogenicity of the organism confirmed that it was a tubercle bacillus of bovine type. It was highly pathogenic to rabbits and guinea-pigs; it was not pathogenic to rats, mice or fowl. An intravenous dose of 0·001 mg. produced widespread tuberculosis in a rabbit in three months. A dose of 0·01 mg. intravenously killed another rabbit in four weeks; infection spread from this rabbit to one in the next cage, which died a week after its neighbour. The lesions in rabbits were severest in the lungs and spleen; the histology was typical of acute tuberculosis. In guinea-pigs the spleen and liver were most heavily involved and showed extensive necrosis ; the degree of splenomegaly was unusually great, the highest weights being 66, 63, 50 and 44 g. (normal: 5 to 10 g.).

DISCUSSION

Dunkin, Laidlaw and Griffith (1929) aptly likened the degree of intracellular bacillosis in tuberculous ferrets to that of Johne's disease and leprosy. Most of the organisms in our spontaneously infected ferret were intracellular, even in the massive foci of coagulative necrosis; extracellular organisms were found mainly in caseous areas. As only the extracellular organisms showed presumptive evidence of lessened vitality (diminished acid-and-alcohol-fastness) it seemed that the intracellular environment provided optimal conditions for the bacilli. In our experimentally infected ferret all the bacilli were intracellular, and bacillus-containing histiocytes were found in every organ and tissue except the brain and spinal cord.

In relation to the macroscopical findings in our second ferret it is interesting that Sprehn (1951) described the spleen and liver of most tuberculous minks as greatly enlarged and usually free from visible tubercles, although obvious lesions are present in other organs in at least half of these cases. Similarly, Dunkin, Laidlaw and Griffith (1929) observed that the spleen was very large in ferrets infected with human tubercle bacilli by subcutaneous inoculation, although

neither organisms nor lesions were found microscopically in this organ.

The histological picture in the liver, spleen and bone marrow of our experimentally infected ferret reminded us forcefully of the picture of certain reticuloses in man. Pathologically, indeed, it would be justifiable to describe the condition as an infective histiocytic reticulosis (reticuloendotheliosis). While the absence of cytopleomorphism distinguished the picture from such conditions in man as Hodgkin's disease, and from certain experimental reticuloses which Gillman, Gillman and Gilbert (1949) produced in rats by repeated intravenous administration of trypan blue, it would be more difficult to differentiate it from that of the so-called storage reticuloses such as Gaucher's disease, or from Letterer-Siwe disease.

We have no adequate knowledge of the normal amount of extramedullary haemopoietic tissue in ferrets, and therefore we cannot assess the extent to which this tissue in our tuberculous ferrets represented a compensatory phenomenon secondary to the replacement of bone marrow by histiocytes.

Regarding the pulmonary lesions in both ferrets, it is relevant that macroscopically identical, yellowish nodules are commonly found in the lungs of non-tuberculous ferrets dying of distemper (Dunkin and Laidlaw, 1926) and in apparently healthy ferrets. These nodules are lipogranulomata, and differ from those in our tuberculous ferrets only in the absence of tubercle bacilli ; it is probable that the nodules in the lungs of these two animals were also lipogranulomata, aetiologically unrelated to the tuberculous infection and only secondarily infected with tubercle bacilli. Similar lesions have been described in the lungs of dogs following intravenous injections of cholesterol (Osborn, Womack, Daum and Kridelbaugh, 1951). Their aetiology in the ferret is obscure; hyperlipaemia of dietary origin or inhalation of droplets of milk-fat seem to be unlikely factors as we have observed no correlation between the fat-content of the diet and their occurrence.

It is interesting that the first sign of illness in each animal was paralysis, manifested first in the adductor muscles of the thighs and rapidly spreading to other muscles. Not unexpectedly, the severity of the disturbance in function was proportionately very much greater than the degree of the demonstrable changes in the anterior horn neurones of the spinal cord and in the muscles. There was no evidence that the paralysis was due directly to tuberculosis; its cause remains debatable. A similar adductor paralysis was mentioned by Yersin (1888) in his classical communication on experimental tuberculosis in rabbits.

SUMMARY

A case of spontaneous tuberculosis in a ferret (*Mustela furo* L.) is described. Histiocytosis, necrosis and bacillosis were the outstanding histological features.

A ferret which was experimentally infected with the organism isolated from the spontaneous case developed great enlargement of the liver and spleen. The histological appearances were those of an infective histiocytic reticulosis and were reminiscent of certain malignant reticuloses in man. There was no necrosis.

The organism was a highly pathogenic tubercle bacillus of bovine type. Its even distribution in smears, without the formation of coils, was atypical. It was strongly Gram-positive when stained by the ordinary rapid Gram method at room temperature.

The literature concerning tuberculosis in the ferret and other Mustelidae is reviewed briefly.

Attention is drawn again to the absence of macroscopically evident disease in some tuberculous ferrets and the resulting risk of transmitting the infection unwittingly by experimental procedures or in vaccines made with ferret tissues. The disease is preventable.

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LEGENDS TO ILLUSTRATIONS

Fig. 1. *Spontaneously infected ferret.* A mesenteric artery and vein and their branches remain virtually intact although surrounded by tissue showing coagulation necrosis. Dead (palely stained) and surviving histiocytes are seen in the necrotic areas. (Mayer's haemalum and eosin; × 90).

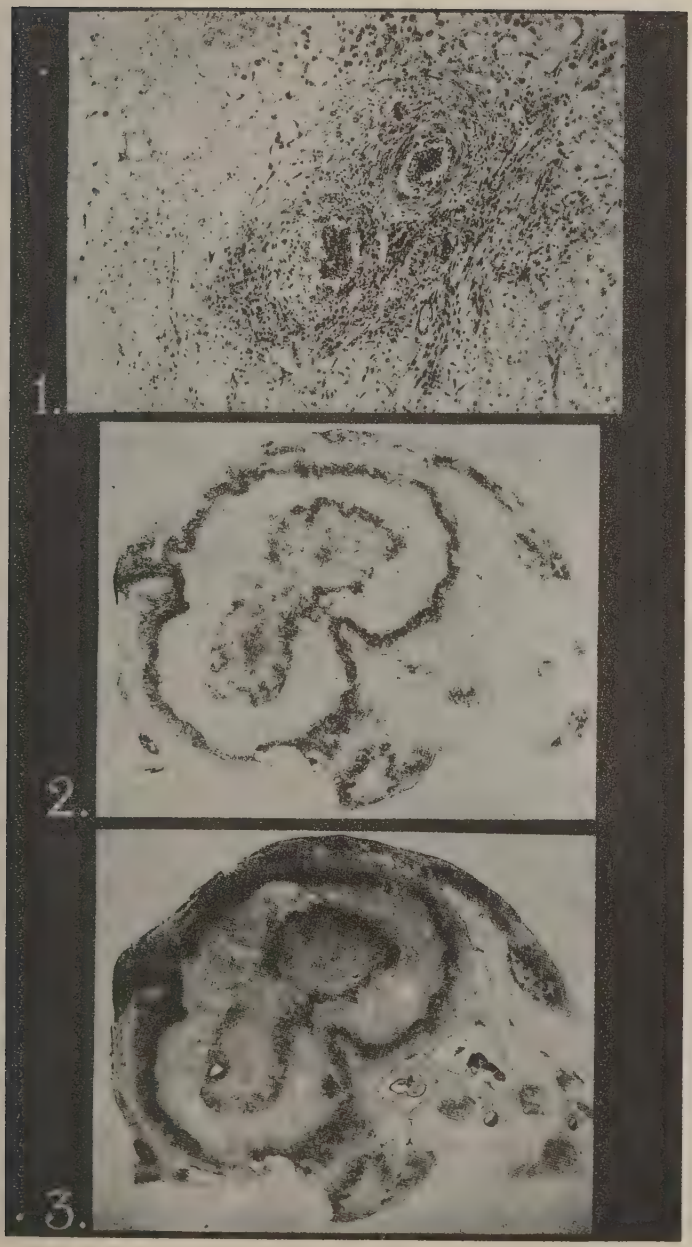
Fig. 2. *Spontaneously infected ferret.* Mesenteric lymph node stained by Ziehl-Neelsen method. The areas of densest concentration of acid-fast bacilli appear black. The node is almost completely necrotic. (× 5)

Fig. 3. Section adjacent to that shown in Fig 2, stained by routine Gram method. Gram-positive bacilli are responsible for the darkly stained areas which correspond with the distribution of acid-fast bacilli in Fig. 2. (× 5)

Fig. 4. *Spontaneously infected ferret.* Greatly dilated lymphatic beneath serosa covering pancreas, the edge of which is seen below. Bacilli visible as black rods in serosal cells (top left) and in the clump of macrophages in the lymphatic. (Ziehl-Neelsen stain and Mayer's haemalum; × 250)

Fig. 5. *Experimentally infected ferret.* Diffuse histiocytosis of liver with extensive replacement of hepatic parenchyma; portal tracts in upper right and lower left quadrants of photograph. (Mayer's haemalum and eosin; × 55)

Fig. 6. *Experimentally infected ferret.* Diffuse histiocytosis of splenic pulp. Most of the small dark cells are erythrocytes. (Mayer's haemalum and eosin; × 115)



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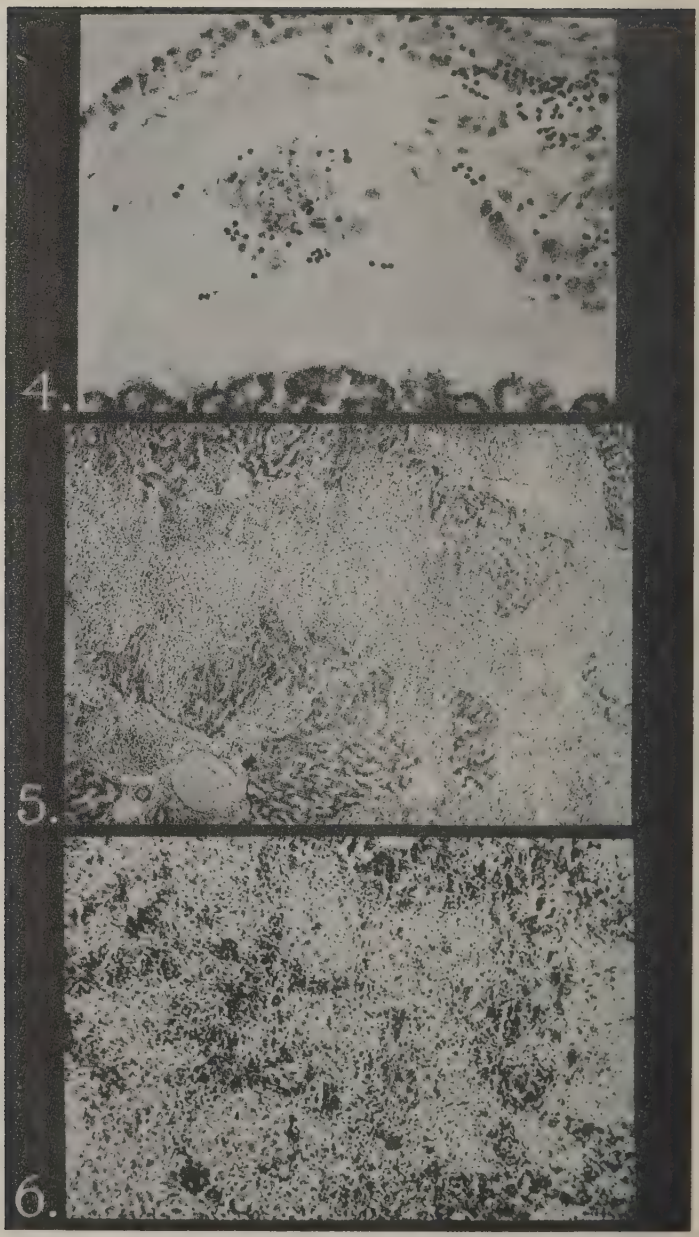

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OBSERVATIONS ON THE TOXICITY OF SALT FOR YOUNG CHICKENS

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INTRODUCTION

Investigations into the toxic effects of sodium chloride when included in poultry mashes suggest that recently hatched chicks are much less tolerant to salt than are mature fowls.

Blaxland (1946) fed mash containing 5, 10, 15 and 20 per cent. of salt for periods of 4 weeks to mature fowls without the development of acute toxic effects: in addition, growing birds, aged 2 to 3 months, did not show excessive mortality, when fed on mash containing 5, 10 and 15 per cent. of salt. Recently hatched chicks, however, showed heavy mortality when receiving mash containing 5 and 10 per cent. of salt. In earlier experiments, Mitchell, Card and Carmen (1926) reared chickens from 9 to 21 weeks of age on mashes containing up to 8 per cent. of salt without detrimental effects to rate of growth or to body condition.

With baby chicks, there would appear to be some degree of variability in the response to salt recorded by different workers. Quigley and Waite (1932), working with newly hatched chicks and salt levels in the food of up to 15 per cent., found that mortality was not excessive until the mash contained 8 per cent. or more of salt and that recently hatched chicks could tolerate salt levels of 30 per cent. for short periods without permanent injury. On the other hand, Sjollema (1935) noted that 2 per cent. salt was injurious to baby chicks. Krakower and Goettsch (1945) fed a mash containing 3 per cent. of added salt to groups of chicks 2 days and 3 weeks old respectively. In the younger chicks, deaths occurred between the 2nd and 34th day, with a peak mortality on the 10th day, including three birds killed because of advanced oedema: there were no survivors out of a total of eighteen birds after 34 days. In the groups of 3 week old chicks offered the same mash, six birds out of a total of fifteen died; eight of the survivors showed no appreciable oedema during the experimental period or at autopsy.

Barlow, Slinger and Zimmer (1948) reared chicks from hatching to 9 weeks of age on diets containing from 0 to 10 per cent. added salt. They also studied the effects of sudden increase of dietary salt on groups of chicks 3, 6 and 9 weeks of age by changing from a mash containing 1 per cent. of salt to diets containing 5 and 10 per cent. Their results suggested that the minimum toxic level was 3 per cent.; with this they obtained an 18 per cent. mortality in a 9 week period. With higher salt levels the number of deaths increased, though not sharply, with a 60 per cent. death rate in 9 weeks when the diet contained 10 per cent. of salt. In the series where the salt level was changed at different ages, the results showed that the toxic effect of salt decreased with increasing maturity, mortality being heaviest in the first month of life.

Salt administered in the drinking water would appear to be more precise in its effects than salt in the mash. Blaxland (1946) found a 100 per cent. mortality in five days when baby chicks ingested 0.9 or 2.0 per cent. sodium chloride solutions. Similar results were obtained by Selye and Stone (1943), when salt solutions replaced drinking water; 2 per cent. solution resulted in death within 3 days, 0.9 per cent. solution was also extremely toxic, but 0.3 per cent. was safe. In a further study, Selye (1943) reported rapid deaths, with obvious signs of generalised tissue oedema, in 2-day old chicks given 2 per cent. solution as drinking water. With older chicks (19 days) some birds receiving 0·9 per cent. solution survived after 20 days, but most developed marked clinical symptoms of muscular weakness, gasping respirations, diarrhoea, cardiac dilatation, oedematous kidneys and ascites which in some cases enormously distended the body cavity. Krakower and Goettsch (1945), found that when a 0.9 per cent. solution replaced the drinking water, 3-week old chicks gave a picture similar to that shown by 2-day old chicks on a mash containing 3 per cent. added salt. Out of fourteen birds, only three survived beyond the third week. Oedema was characteristic and was accompanied by a considerable elevation of plasma chloride and a reduction in total plasma protein. Hypertrophy of the heart and the kidney glomeruli usually followed excessive intakes of salt administered either in mash or in drinking water. The toxic effects of large amounts of salt in mash or drinking water were ascribed to the associated high water consumption, since at high salt levels the amount of fluid absorbed tended to exceed the birds' tolerance. Further work by Krakower and Heino (1947), on the cardiorenal changes associated with high salt intakes, showed that the daily amounts of sodium chloride ingested must exceed 0.3 g. per 100 g. body-weight if morphological changes in the heart and the kidney were to occur. With this level of sodium chloride, the daily water consumption exceeded 50 cc. per 100 g. live weight. Cardiac hypertrophy was a constant feature with high salt, whereas renal hpertrophy appeared to be associated with a progressive increase in the salt intake, glomerular enlargement occurring only where body growth was possible i.e. where calorie restriction was not so excessive as to prevent growth and where age was not a limiting factor.

It was the primary purpose of this investigation to study the effects of mash containing relatively low levels of salt on the health of newly-hatched chicks, as experience in the field led to the suspicion that lower levels of salt than those usually accepted as toxic, could, on occasion, be associated with high death rates in very young chicks. The relationship between salt and water intakes also appeared to be of importance and this factor was also studied.

METHODS

Day-old chicks (L.S. × R.I.R., males) were divided into groups of six and housed in small wire pens in an electrically heated room fitted with thermostatic control. The temperature could be adjusted to 90° F. at the outset and gradually reduced to 70° F. over a period of three weeks. By this means it was possible to keep a number of small groups of young chickens under similar conditions.

Food and water were always available, losses due to evaporation of water being assessed by controls. The weights of each group of chicks and the amounts of food and water consumed were recorded daily.

A preliminary experiment, in which two groups of six chicks were kept under these experimental conditions on the food to be used as a basal diet, showed the system to be satisfactory; the chicks grew normally, the average gain per chick over the experimental period of 20 days being 140 g. No deaths occurred, all birds appearing perfectly healthy throughout.

The basal diet consisted of :—

Bran	23	per cent.
Sussex Ground Oats	22.5	„ „
Maize Gluten	20	„ „
Palm Kernel Cake	7·5	„ „
Meat and Bone Meal	7·5	„ „
Dried Milk	6.25	„ „
Dried Potato	5	„ „
Sesame Cake Meal	2.5	„ „
Herring Meal	2.5	„ „
Ground Limestone	1.25	„ „
Cod Liver Oil	1.25	„ „

The moisture content of this ration was 12·5 per cent., the chloride 0·52 per cent. Cl, corresponding to a level of 0·98 per cent. NaCl in the dry matter.

RESULTS

DAY OLD CHICKS

Experiment I. (NaCl per cent = 0·98 to 3·25 over an experimental period of 20 days).

Ten groups of six chickens were used to compare levels of salt ranging from 0·98 to 3·25 per cent. NaCl with increments of 0·25 per cent. throughout the range. The diets were prepared by adding the requisite calculated amounts of salt to a weighed amount of food followed by a thorough mixing, the salt concentration being checked by analysis using the method of Husband and Godden (1927).

Health of the chicks. No deaths occurred in any of the groups, and of the sixty chickens used, only two showed signs of abnormality. One chicken in the highest salt group (3·25 per cent. NaCl) showed a disinclination to eat on the twelfth day, and appeared rather drowsy with a tendency to gape, but made a complete recovery later in the experiment. One chicken in the lowest salt group developed leg weakness which may have been due to trauma. Apart from

those two, all the groups remained healthy, showing normal feather development and a good appetite.

Rate of growth. To the consecutive daily weights of the ten groups of chicks, reduced to mean weights per chick, second degree curves have been fitted (Wishart, 1938).

TABLE I

EXPERIMENT 1. AVERAGE RATE OF GROWTH

Group No.	*Percentage NaCl in Dry Matter of Ration*	*Wo*	*g.*	*h.*	g_1
0	0·98	41·3	8·08	0·316	8·14
1	1·23	40·5	7·49	0·352	7·94
2	1·48	41·0	8·35	0·326	7·90
3	1·74	37·5	7·26	0·308	7·34
4	1·99	38·3	7·36	0·262	7·43
5	2·24	39·7	7·76	0·262	7·43
6	2·49	41·3	7·42	0·275	7·33
7	2·74	36·3	6·80	0·251	6·77
8	3·00	38·3	6·89	0·275	6·93
9	3·25	42·2	7·28	0·228	7·34
Average:	2·11	39·6	7·47	0·285	7·47

"Wo" is the initial weight in g. per chick, "g" is the average growth rate in g. per day, "h" is half the rate of change of growth rate in g. per day, "g_1" is the average growth rate after correction for the variations in initial weight, by calculation of the partial regression equation.

It will be seen from Table I that the average growth rate shows a tendency to fall with increasing concentrations of sodium chloride. The regression equation for the average growth rate " g " on the percentage of NaCl in the food shows that for unit increase in the salt percentage there is a decrease in the average growth rate of 0·44 g. per day. This regression coefficient is just statistically significant ($P = 0{\cdot}05$). The partial regression coefficient (—0·416) after correcting for variation in initial weight, is also significant at the 5 per cent. level. The regression of " h ", half the rate of change of growth rate on the percentage of salt in the food, is significant at the 1 per cent. level. This decrease in curvature with increase in percentage salt indicates an apparent stimulus to growth in the early stages at the higher salt levels, although the stimulus was not maintained later in the experiment.

Food and water consumption. Table II gives the average daily intake of water, food and salt per bird and per 100 g. body weight over the whole experimental period.

TABLE II

AVERAGE DAILY INTAKE OF FOOD, WATER AND SALT OVER THE WHOLE PERIOD

1.	2.	3.		4.		5.		6.
% *NaCl in dry matter*	*Av. Wt. (g. per Chick)*	*Av. Daily Food Intake (g. D.M.)*		*Av. Daily Water Intake (g.)*		*Av. Daily Salt Intake (g.)*		*Ratio Water/Food*
		Per Chick	*Per 100 g. B.W.*	*Per Chick*	*Per 100 g. B.W.*	*Per Chick*	*Per 100 g. B.W.*	
0·98	95·5	16·7	17·5	38·5	40·3	0·164	0·175	2·31
1·23	89·2	16·6	18·6	40·8	45·7	0·204	0·229	2·46
1·48	97·1	17·0	17·5	42·6	43·9	0·252	0·259	2·52
1·74	86·2	16·2	18·8	44·3	51·4	0·282	0·327	2·74
1·99	89·9	15·7	17·5	44·7	49·7	0·344	0·348	2·81
2·24	92·7	17·4	18·8	54·7	59·0	0·390	0·421	3·15
2·49	88·1	15·6	17·7	50·8	57·7	0·388	0·441	3·26
2·74	85·2	16·3	19·1	54·3	63·7	0·447	0·523	3·41
3·00	87·3	15·3	17·5	52·6	60·3	0·459	0·525	3·44
3·25	97·8	17·3	17·7	67·8	69·3	0·562	0·575	3·92
2·11	90·9	16·4	18·1	49·1	54·1	0·349	0·382	3·00

It will be noticed that the increase of salt in the ration has no significant effect on the average daily amount of food consumed. Water intake, on the other hand is considerably greater in the higher salt groups than in the lower. The regression of water intake per unit intake of food (Column 6), on percentage of salt in the ration shows that for every unit increase in the salt percentage, the water intake per 100g. of food increases by 62.2 g. and that the water intake for 100 g. of salt-free food is assessed as 169·6 g.

The average daily intakes of food, water and salt per 100 g. body weight are considered in consecutive periods of four days, giving five periods, in all, viz. P_1, P_2, P_3, P_4 and P_5 respectively (Table III). It will be seen that the relative food and water intakes during the first four days of life are approximately half of the requirement during subsequent periods and that from the age of five to twenty-one days, the requirements per 100 g. body weight remain relatively constant.

The water requirements per unit intake of food show a well marked trend with increasing salt intake and are not markedly different for the different periods; during the first period, the requirement per unit food intake at all levels of salt is somewhat higher than in subsequent periods. From this experiment, the general conclusion may, therefore, be drawn that chicks under 3 weeks of age can tolerate a ration containing up to 3 per cent. of salt without showing any marked harmful effects apart from a slight decrease in growth rate at the higher levels. The intake of increased amounts of salt is accompanied by an additional intake of fluid

which is presumably necessary for the kidneys to function adequately and prevent any accumulation of salt in the tissues. The excreta became more moist with increasing salt concentration, but owing to the amount of evaporation which occurs at the high environmental temperatures employed, it was impossible to get any satisfactory measure of the fluid output.

TABLE III

AVERAGE DAILY INTAKE PER 100 G. BODY WT. TAKEN OVER FIVE CONSECUTIVE PERIODS OF FOUR DAYS

NaCl %		*0·98*	*1·23*	*1·48*	*1·74*	*1·99*	*2·24*	*2·49*	*2·74*	*3·00*	*3·25*
Food,	P_1	9·0	9·4	9·5	9·9	9·8	10·4	7·7	9·2	9·9	10·2
Dry	P_2	18·2	20·4	19·4	19·6	17·8	19·5	18·3	18·0	17·8	17·3
Matter).	P_3	19·4	21·9	19·3	20·7	19·3	22·8	18·7	21·1	17·7	19·7
(g.)	P_4	18·9	19·7	18·2	20·1	18·3	19·3	19·3	19·9	18·3	18·6
	P_5	17·6	18·2	17·2	19·0	18·2	21·1	18·7	19·4	18·9	18·2
Water,	P_1	23·5	27·8	27·4	29·8	33·1	37·9	30·7	35·2	43·2	39·7
(g.)	P_2	43·0	49·2	49·1	54·3	57·5	62·6	62·4	68·0	69·1	71·6
	P_3	41·5	52·3	49·6	56·9	55·0	67·3	61·6	75·3	58·4	80·0
	P_4	44·0	50·0	42·8	55·5	47·9	70·9	63·3	65·3	62·6	71·2
	P_5	40·7	43·3	44·3	50·3	50·6	50·4	58·0	62·7	60·9	69·3
Salt,	P_1	·088	·116	·141	·172	·195	·233	·192	·252	·297	·325
(g.)	P_2	·178	·251	·287	·341	·354	·437	·456	·493	·534	·562
	P_3	·190	·269	·286	·360	·384	·511	·466	·578	·531	·640
	P_4	·185	·242	·269	·350	·364	·432	·481	·545	·549	·604
	P_5	·172	·224	·255	·331	·362	·473	·466	·532	·567	·592
Ratio,	P_1	2·61	2·96	2·86	3·01	3·38	3·64	3·99	3·83	4·36	3·97
Food	P_2	2·26	2·41	2·53	2·77	3·23	3·21	3·41	3·78	3·88	4·14
Water	P_3	2·14	2·39	2·57	2·75	2·85	2·95	3·27	3·56	3·30	4·06
	P_4	2·33	2·54	2·35	2·76	2·62	3·67	3·28	3·28	3·42	3·83
	P_5	2·31	2·38	2·58	2·65	2·78	2·79	3·10	3·23	3·22	3·81

Experiment II. (NaCl percentage = 3·52 to 7·93).

Since no deleterious effects were apparent within the range of salt concentration used in the first experiment, the range was increased in order to determine the level at which pathological effects would occur. The second experiment was carried out on the same lines as the first, but the salt concentration ranged from 3·52 to 7·93 per cent. with increments of 0·49 per cent.

Health of the chicks. A number of fatalities occurred which appeared attributable solely to the presence of the large quantities of salt in the diet (Table IV). The deaths were widely dispersed both in time and in relation to salt concentration, with a slight tendency for earlier deaths at the higher salt levels.

Symptoms. The chickens which died before the tenth day showed symptoms of increasing drowsiness with drooping wings and on

evident desire for food or water; after three to four hours they were unable to stand, and death ensued a few hours later. No oedema was noticeable nor was there any obvious tendency to gape. As the chickens became older the symptoms became more acute. Drowsiness characterised all cases and two to three days before death, the majority developed well marked oedema which affected all the body tissues, being especially pronounced along the abdomen. In a few the oedema was slight but they had a marked tendency to gape.

TABLE IV.

Group	*% NaCl in Dry Matter*	*Total Number of Deaths*	*Day of Deaths*
10	3·52	—	
11	4·01	1	14th
12	4·50	3	1 on 10th, 2 on 20th
13	4·99	1	12th
14	5·48	1	18th
15	5·97	3	4th, 9th, 10th
16	6·46	2	6th, 8th
17	6·95	1	16th
18	7·45	3	1 on 10th, 2 on 13th
19	7·93	3	7th, 15th, 17th

The value of the figures for rate of growth is limited because of the deaths which occurred and also by the oedema shown by most chicks at some stage. However, for purposes of comparison with the data obtained in the previous experiments, the values for average growth rate are given in Table V.

TABLE V

EXPERIMENT II. AVERAGE RATE OF GROWTH

Group	*% NaCl in Dry Matter of Ration*	*Wo.*	*g.*	*h.*
10	3·52	42·5	6·98	0·092
11	4·01	41·2	6·61	0·099
12	4·50	41·3	6·79	0·234
13	4·99	39·8	4·73	0·173
14	5·48	39·2	5·01	0·231
15	5·97	39·8	6·43	0·072
16	6·46	45·0	3·85	0·003
17	6·95	41·7	4·03	0·066
18	7·45	39·2	4·21	0·065
19	7·93	38·7	3·68	0·028
Mean	5·73	40·8	5·23	0·106

The decrease in growth rate with increasing salt concentrations in the ration, noticed in the first experiment, is again in evidence, the effect being more marked at the higher levels. The irregularities of the rate of change of growth rate are mainly due to the deaths.

Food and water consumption. The results obtained in this second trial are summarised in Tables VI and VII and are again affected to some extent by the deaths during the experimental period. The

TABLE VI

AVERAGE DAILY INTAKE OF FOOD, WATER AND SALT OVER THE WHOLE PERIOD

% *NaCl in Dry Matter*	*Av. Wt. (g. per chick)*	*Av. Daily Food Intake (g. D.M.)*		*Av. Daily Water Intake (g.)*		*Av. Daily Salt Intake (g.)*		*Ratio Water/Food*
		Per Chick	*Per 100 g. B.W.*	*Per Chick*	*Per 100 g. B.W.*	*Per Chick*	*Per 100 g. B.W.*	
3·52	97·6	17·0	17·4	72·6	74·3	·598	·613	4·27
4·01	94·9	16·5	17·4	65·9	69·4	·662	·697	3·99
4·50	89·8	15·2	16·9	68·1	75·8	·684	·762	4·48
4·99	76·9	13·7	17·8	57·9	75·2	·684	·910	4·23
5·48	75·4	13·2	17·5	58·7	77·8	·723	·959	4·45
5·97	94·4	14·0	14·8	73·2	77·5	·836	·885	5·23
6·46	79·8	14·7	18·4	61·4	77·0	·949	1·190	4·18
6·95	76·4	12·9	16·9	66·0	86·4	·897	1·174	5·11
7·45	76·3	13·1	17·2	78·9	103·4	·976	1·280	6·00
7·93	73·8	15·3	21·2	66·8	90·5	1·214	1·644	4·35
5·73	83·5	14·6	17·6	66·9	80·7	·822	1·011	4·63

food intakes, however, are fairly constant from group to group when assessed on a basis of uniform body-weight. The water intake shows an upward trend as the concentration of salt increases but the effect is much less marked than in the first experiment. The regression for the water requirement per unit intake of food (Table VI, Col. 6) on percentage NaCl in the ration shows that for the range of salt concentration of 3·52 per cent to 7·93 per cent, there is an increase in water intake of 23·3 g. for each additional gramme of salt consumed. This may be compared with the figure of 62·2 g. water for each gramme of salt consumed in Experiment I. The extrapolated value of 329·7 g. of water for 100 g. of food for the intake with food containing no salt contrasts also with the value of 169·6 g. found in Experiment I. That this difference is not due to lack of continuity between the two experiments is readily seen from the ratios of water/food; the difference is almost entirely due to differences in slope of the curves in the respective salt ranges.

TABLE VII

AVERAGE DAILY INTAKE PER 100 G. BODY WT.

NaCl % on D.M.		*3·52*	*4·01*	*4·50*	*4·99*	*5·48*	*5·97*	*6·46*	*6·95*	*7·45*	*7·93*	*Mean*
Food, (g. Dry Matter)	P_1	10·1	9·1	9·2	8·5	9·1	10·7	8·3	8·2	8·6	9·0	9·08
	P_2	18·2	15·1	17·6	14·1	14·9	14·7	12·3	13·3	16·7	15·6	15·2
	P_3	20·2	19·9	19·1	17·2	17·9	16·7	19·4	19·9	19·4	17·9	18·7
	P_4	16·6	17·7	17·9	19·2	17·3	13·1	19·8	15·9	15·5	23·8	17·6
	P_5	18·4	19·3	17·0	22·5	21·6	16·4	24·5	21·6	21·1	28·6	21·0
Water, (g.).	P_1	49·9	43·7	52·7	52·9	63·1	64·2	53·6	55·4	66·8	65·4	56·7
	P_2	85·3	62·9	67·0	70·3	86·0	85·3	76·8	75·2	108·9	82·8	81·8
	P_3	83·7	71·9	88·2	76·1	79·5	76·2	86·4	101·7	118·8	76·5	85·9
	P_4	71·9	75·0	81·2	78·7	78·5	80·5	83·5	89·3	109·1	84·8	86·1
	P_5	73·5	74·4	67·1	83·1	78·0	77·1	75·0	93·9	109·3	113·0	84·4
Salt, (g.)	P_1	·355	·365	·414	·424	·499	·639	·536	·570	·641	·714	·515
	P_2	·641	·606	·792	·703	·817	·878	·795	·924	1·244	1·237	·862
	P_3	·711	·798	·860	·858	·981	·997	1·253	1·383	1·445	1·419	1·070
	P_4	·584	·710	·806	·958	·948	·782	1·379	1·105	1·155	1·887	1·021
	P_5	·648	·774	·765	1·123	1·184	·979	1·583	1·501	1·572	2·268	1·239
Ratio, Food Water	P_1	4·94	4·80	5·73	6·22	6·93	6·00	6·46	6·76	7·77	7·27	6·28
	P_2	4·69	4·16	4·94	4·99	5·77	5·80	6·24	5·65	6·40	5·31	5·39
	P_3	4·14	3·61	4·62	4·43	4·44	4·56	4·46	5·11	6·12	4·27	4·57
	P_4	4·33	4·24	4·54	4·10	4·54	6·15	4·22	4·62	7·04	3·98	4·87
	P_5	3·99	3·85	3·95	3·69	3·61	4·70	3·06	4·35	5·18	3·95	4·03

The values for the ratio water/food given in Table VII where the results are summarised in five consecutive four-day periods, are of interest in demonstrating that during early periods, the chickens show a fair capacity to maintain the additional water intake required by the increasing salt concentration. In the last period of four days the ratios tend to be uniform throughout the salt range, indicating a fall in either the ability or the need to ingest water in proportion to salt with increasing age.

In the first period of four days, the food intake over the whole range of salt concentrations was approximately half the value for the subsequent periods when calculated on the basis of 100 g. body weight, so that the salt intake during this period is relatively low in all the groups. The full toxic effect of a ration high in salt is not likely to be shown until maximum food intake per 100 g. body weight has been attained. There was only one death in the first period of four days and this occurred on the fourth day with a concentration of salt in the ration of 5·97 per cent.

Experiment III. (NaCl per cent = 1·74 to 3·25 and restricted water).

In view of the possibility that under field conditions deaths might occur at levels of salt below 3·5 per cent. if inadequate amounts of drinking water are supplied, an experiment was carried out in

which the conditions of the first experiment were reproduced but the water intake was restricted, so that it remained approximately constant throughout the salt range. Only four levels of salt concentration were compared in this experiment, viz., 1·74 per cent., 2·24 per cent., 2·74 per cent. and 3·25 per cent. expressed as before on a dry matter basis (Groups 20 to 23). The experimental technique followed, apart from the limitation of water intake, was exactly the same as in Experiments I and II.

The average daily intakes of food, water and salt compared with the corresponding values from Experiment I are given in Table VIII.

TABLE VIII

A. RESTRICTED WATER (DATA FROM EXPT. III)

% *NaCl in Dry Matter*	*Av. Wt. (g. per chick)*	*Av. Daily Food Intake (g. D.M.)*		*Av. Daily Water Intake (g.)*		*Av. Daily Salt Intake (g.)*		*Ratio Water/Food*
		Per Chick	*Per 100 g. B.W.*	*Per Chick*	*Per 100 g. B.W.*	*Per Chick*	*Per 100 g. B.W.*	
1·74	87·0	15·2	17·5	40·8	46·9	·264	·304	2·68
2·24	89·0	15·6	17·5	42·3	47·7	·349	·392	2·72
2·74	84·1	14·5	17·2	43·6	51·8	·397	·472	3·00
3·25	32·0	13·8	16·8	46·1	56·2	·448	·547	3·34

B. UNRESTRICTED WATER (DATA FROM EXPT. I)

1·74	86·2	16·2	18·8	44·3	51·4	·282	·327	2·74
2·24	92·7	17·4	18·8	54·7	59·0	·390	·421	3·15
2·74	85·2	16·3	19·1	54·3	63·7	·447	·523	3·41
3·25	97·8	17·3	17·7	67·8	69·3	·562	·575	3·92

Absolute control of the water intake was difficult to achieve. It will be noted from the table that there was a minor increase as the salt level was increased, with 40·8 g. as the average daily intake at the lower level of 1·74 per cent. NaCl and 46·1 at the 3·25 per cent salt level. Comparison with the water intakes when the supply was unrestricted, viz. 44·3 g. increasing to 67·8 g. at the higher level, indicates, however, that a considerable measure of control of water intake was obtained.

In the group receiving food containing 3·25 per cent. NaCl there was only one death on the 8th day apparently due to heart failure; no marked oedema was present.

In the groups with an appreciable restriction of the water intake, when the daily supply of water was exhausted, the birds became so noisy that it would seem most unlikely that this set of circumstances could arise in commercial practice without the cause of the trouble becoming apparent.

The food intakes and growth rates were appreciably affected by the limit on the amount of water available; when the daily supply of water was exhausted, the appetite became negligible.

The values for the average growth rate " g " compared with values obtained for the first experiment were :—

% NaCl in D.M.	*Restricted Water* "g"	*Unrestricted Water* "g"
1·74	5·86	7·26
2·24	6·09	7·76
2·74	5·94	6·80
3·25	5·90	7·28

GROWING CHICKS

Increasing salt concentrations

This experiment commenced on February 27th with 14 chickens, 28 days old. They received a mash containing 1·96 per cent. of added sodium chloride : within 24 hours water consumption increased and the birds showed a tendency to scour which became very evident by March 4th. The added salt was increased on the 5th, 7th, 15th and 18th of March to give concentrations of 2·64, 3·85, 4·76 and 5·66 per cent. respectively. No deaths occurred during this period of 20 days except for two birds killed for post-mortem on March 18th

On 19th March the chicks were split into two groups of six each. One lot was maintained on the ration containing added salt and the other, kept as controls, was fed on ordinary " growers' " mash. Between the 19th and 22nd March the concentration of added salt was 5·66 per cent., this being increased to 6·54 per cent. on the 23rd and maintained at this level until the 25th.

Birds receiving this ration were looking anaemic, dirty and were not so lively as the controls. Water consumption of the two groups was compared on the 22nd and again on the 25th, 6 pints being used per day by the salt-receiving group, compared with 3 pints for the controls. On the 26th the concentration was increased to 7·41 per cent and maintained at this level until April 2nd. One chicken appeared droopy on the 30th but recovered during the following day. On April 3rd the salt was increased to 9·09 per cent and maintained at this level for six days.

On April 8th three birds from each group were killed for post-mortem examination (*see* later). The salt was increased to 10·71 per cent. on the 10th and was further increased to 13·05 per cent on the 19th. On April 24th, after 56 days on experiment, the added salt became 16·16 per cent. On the 25th no deaths having occurred the birds were weighed and the water consumption measured. The three birds on the high salt ration were 2 lb. 12 oz., 2 lb. 7 oz. and 2 lb. 15 oz. compared with 3 lb. 9 oz., 3 lb. 12 oz. and 3 lb. 3 oz.

for the controls. Water consumption was 7½ pints for the salt group and 2 pints for the controls.

On 26th April, one of the chickens receiving the ration containing 16·16 per cent salt died, the chief finding at post-mortem being a grossly enlarged heart.

During the following month the salt content of the ration fed to the two remaining chickens was further increased, viz. to contain 20 per cent on May 1st, 23 per cent on the 8th, 28 per cent on the 18th, 33 per cent on the 23rd and 37·5 per cent on the 29th. Both chickens were able to tolerate these exceptionally high concentrations of salt, although on the 2nd one bird with 20 per cent. added NaCl, was staggering but recovered the next day. The weights of the birds on the 27th were 3 lbs. for both birds receiving salt and an average of 4 lbs. 10 oz. for the controls.

During the next month the salt concentration was increased from 37·5 to 50 per cent. After being on the 50 per cent. ration between the 21st and 26th June, the salt concentration was gradually reduced to reach 33 per cent. on July 8th. The two birds were maintained at this level, the weights being recorded periodically, until July 27th when the experiment was discontinued. The weights on this date were 3 lb. 4 oz. and 3 lb. 12½ oz. compared with a mean weight of 6 lb. 9 oz. for the controls.

POST MORTEM FINDINGS

Macroscopic

There was a marked variation in the lesions in chicks which died during the first nine days of the experiment compared to those which died after that time, irrespective of the percentage of salt they were receiving.

In the early deaths there were no characteristic changes. The crop usually contained fluid and the gizzard a varying quantity, usually small, of moist food material. The kidneys were slightly enlarged and somewhat paler than normal and some presented a slightly mottled appearance. The surface was smooth and no alteration in shape was noted. In nearly every case the ureters contained quantities of whitish chalky material. The intestinal contents were fluid and the food had the appearance of not being properly digested. There was no evidence of enteritis.

In chicks which died between the tenth and twentieth days a completely different picture was obtained. Extensive oedema was a striking and constant feature in every case except one. There was frequently a considerable accumulation of fluid in the subcutaneous tissue in the neck region and over the thorax which extended to the lower abdomen and in some cases to the limbs. The flesh presented a moist, glistening appearance and ascites was commonly noted. In every case the heart was enlarged, in some slightly, in others up to twice the normal size. This striking lesion was often associated

with oedema of the lungs and a varying degree of hydropericardium was usually present. In most cases there was no apparent increase in size of the kidneys which were as a rule paler in colour than normal. In one bird, receiving 4·01 per cent. salt, the kidneys were congested with small haemorrhages, and in another receiving 4·5 per cent. salt, they were swollen, purplish in colour with extensive subcapsular haemorrhages. In both cases there was an associated congestion of the liver and the coronary and mesenteric blood vessels were engorged. The crop and gizzard were almost invariably empty; the contents of the small intestine were dry and in several cases there was subacute impaction. The caecal contents were usually fluid; in some cases distension was marked. Considerable diversity of lesions was noted in chicks from the same group, e.g., two chicks receiving 4·5 per cent. of salt died on the same day. In one there was extensive oedema accompanied by a great enlargement of the heart; the kidneys were swollen and presented a congested, purplish appearance, with secondary changes in the liver. In the other, oedema was scanty and the heart was only slightly enlarged. The kidneys were yellowish-grey in colour and bloodless in appearance. In both cases there was sub-acute impaction of the small intestine and the caeca were distended with fluid.

Twenty-two days after the experiment had started, eighteen of the survivors were destroyed. In eight cases they appeared to be normal healthy chicks, and in two others the only abnormality noted was an unusually dry condition of the intestinal contents. The apparently healthy birds were in groups 13, 14, 15, 17, 18, 20 and 23.

In the eight chicks remaining there was a varying degree of oedema in every case accompanied by hypertrophy of the heart, in one case massive in extent. In seven of these birds the caeca were distended with fluid; in three there was a sub-acute impaction of the small intestine and in one the cloaca was impacted.

Microscopic

The most striking feature in the kidneys was hypertrophy of the glomeruli. This was most marked in the medulla and was sometimes confined to this region, but in other cases the cortical glomeruli were also involved. The glomeruli were sometimes greatly enlarged, excessively cellular and presented a lobulated, bulging, irregular appearance, instead of the normal circular shape. In the more advanced cases these changes varied greatly in adjacent lobules, some of which appeared almost normal. Some hypertrophy of Bowman's capsule accompanied by marked hyalinisation was noted. In some cases the glomeruli were almost completely replaced by fibrous tissue. Tubular changes were not marked; cloudy swelling sometimes occurred and tubular casts were numerous. Interstitial oedema was sometimes present and accumulations of red blood cells

were noted in the interstitial tissue. There was no evidence, however, of any increase in interstitial connective tissue and no arteriosclerotic changes, such as are present in mammalian nephritis, were seen in any of our cases.

At first sight the kidney changes were suggestive of acute and sub-acute glomerulo-nephritis. An examination of a number of kidneys, however, from chicks, which had been retained after the feeding of salt was discontinued, showed them to be more or less normal, the glomeruli having apparently resumed their normal size and shape. It would appear, therefore, that glomerular hypertrophy resulted from an attempt to increase the available area of filtration and that the other pathological changes were associated with excessive work imposed on the kidneys by the high water and salt intake and were not indicative of a true nephritis.

Sections of the lungs showed marked oedema, particularly interstitial, although in some cases fluid was present in the alveoli. Oedema of the myocardium was common.

In early cases liver changes were insignificant; in later cases capsular thickening accompanied by atrophy of the liver cells was sometimes seen.

DISCUSSION

The response shown by newly hatched chicks to salt incorporated in the mash is widely variable from bird to bird; some birds succumb when the salt level is of the order of 3·5 to 4·0 per cent.; others survive with 8 per cent. or more. This wide variability in mortality effect would seem to be due to differences shown by individual birds in their capacity to balance the salt intake by a sufficient intake of water. From the results reported in the literature, it would appear that salt in the drinking water is much less variable in its effects than is salt in the mash. The experiments of Krakower and Goettsch (1945) and Krakower and Heino (1947) suggest that a 0·9 per cent. salt solution is approximately equivalent in its toxicity to a salt level in the mash of 3·5 to 4·0 per cent. The approximate salt concentration of fluid available to the bodies of chicks fed on mash containing varying levels of salt can be calculated from the amounts of ingested salt and water given in Tables II and IV. These are as follows:—

% NaCl in Mash ..	0·98	1·23	1·48	1·74	1·99	2·24	2·49	2·74	3·00	3·25
Equal to a										
% NaCl in Fluid of ..	0·42	0·50	0·59	0·64	0·77	0·71	0·76	0·82	0·87	0·83
% NaCl in Mash ..	3·52	4·01	4·50	4·99	5·48	5·97	6·46	6·95	7·45	7·93
Equal to a										
% NaCl in Fluid of ..	0·82	1·00	1·00	1·18	1·23	1·14	1·54	1·36	1·24	1·81

No deaths and little oedema developed in chicks on mash with levels of salt of 3-5 per cent. or less, i.e., where the equivalent salt concentration of ingested fluid was less than 0·9 per cent. Above 3·5 per cent. salt in the mash, where the concentration of salt in the avail-

able fluid was greater than 0·9 saline, deaths were frequent and oedema was noticeable at some stage in most chicks. In this general sense the results with salt in the drinking water and with salt in the mash are comparable. It would seem likely, however, that accommodation to salt in the mash is more readily attainable than to salt in the drinking water since with the former, compensation can be achieved within limits by a lowering of the food intake and an increase in the water intake. No such adjustment is possible with salt solution as the sole source of fluid for drinking. That young chicks do not invariably protect themselves by this means when on mash high in salt may be due to a poor sense of taste, hunger being satisfied: where the salt content is high, the water required to obtain adequate dilution exceeds the birds' capacity. Krakower and Goettsch regard a daily water intake of more than 50 per cent. of the body weight as exceeding the birds' tolerance; our values suggest a value of 70 per cent. for very young chicks. At the highest salt levels birds were ingesting daily their own weight of water and even this amount was insufficient to give the amount required to dilute the ingested salt to physiological concentrations.

The decline in the rate of increase of the water/food ratio with increasing salt shown at the higher salt levels, also indicates that the capacity to ingest sufficient water becomes critical when the salt percentage of the food exceeds 3·5. With 0·98 per cent. salt in the mash, the average value of the water/food ratio is 2·31 and this value increases steadily with increasing salt to give an average value of 4·27 when the mash contains 3·52 per cent. There is little further rise in the ratio as the level is further increased. Krakower and Goettsch 1945), working with birds studied individually and maintained over a six week period at two levels of salt concentration (a normal ration and the same plus 3 per cent. added salt), found a narrow range in the week to week variation of the water/food ratio as shown by individual birds, but a much greater variation between different birds on the same food. The fact that birds behave consistently over a six week period and that there are well marked differences between individual birds on the same mash indicates why some birds survive and others succumb to salted food. Those birds which consistently show a high water/food ratio have the best chance of survival when on high-salt diets.

That 0·9 per cent. salt solution for drinking or its equivalent as salt in the mash may be toxic to newly hatched chicks, is associated with the incapacity of the kidney to preserve osmotic constancy at this stage of life. The excretion of salt and reabsorption of water by the kidney must at least balance the trend towards hypertonicity resulting from the water lost in thermo-regulation. This maintenance of balance would appear to be beyond the capacity of the kidney of the newly hatched chick when the salt concentration of ingested fluid is much more than 0·9 per cent. In this respect it may parallel the newly born mammal, where, if the internal environment is disturbed,

the kidney makes a relatively feeble attempt to restore equilibrium (McCance, 1948). There is a further factor which influences the relative tolerance shown by older birds to salt in the diet. The food requirements of young chicks per 100 grammes body weight are appreciably greater than those of adult birds, while the water intake can apparently, if necessary, bear the same relationship to the body weight in age as in youth. The net effect of these factors is to render a mash containing a dangerous level of salt less toxic to mature birds than to chicks. To illustrate, we can consider the three growing birds on mash with increasing salt concentration which after 57 days on experiment were receiving a mash containing 16·2 per cent. of added salt. The water intake averaged 2·5 pints per bird. The amount of food which could be eaten to give an effective concentration of 0·9 per cent. salt in the available fluid is 2·7 oz. which is only slightly below the standard requirement for birds of this size (Robinson, 1949). These birds on mash containing 16 per cent. salt are in similar circumstance to day-old chicks on a mash containing 4 per cent. salt and for adult birds the same degree of hazard would be obtained with a mash containing 20 to 24 per cent. of salt. The apparent increase in tolerance with age is probably due, therefore, to the normal relative decrease in food requirement as the bird matures.

SUMMARY

In view of conflicting results in the literature, it was thought worth while to make a detailed study of the toxicity of salt in the diet of newly hatched chickens and its effect on growth rate.

Ten groups of chickens on levels of salt ranging from 0·98 to 3·25 per cent. showed no harmful effects apart from a slight decrease in growth rate at the higher levels. Increasing salt in the ration had no appreciable effect on food intake but led to a considerable increase in water consumption.

A similar experiment with salt concentrations ranging from 3·52 to 7·93 per cent. NaCl led to a 30 per cent mortality. The earlier deaths, which occurred mainly at the higher salt levels, were characterised by drowsiness and inability to stand; chickens which lived ten days or longer showed signs of generalised oedema and a tendency to gape. The effects on growth rate and on food and water intake were in line with the results of the previous experiment.

Restriction of water intake in association with moderately salty diets led to a compensatory reduction of food intake.

Increasing the salt concentration with chickens over 1 month old gradually from 2 per cent. to 50 per cent. interfered greatly with growth but was not necessarily fatal. This was probably associated with the lower food requirements of adult birds in relation to live-weight and possibly also with increased renal efficiency in the older birds.

Apart from the strikingly extensive oedema shown by chickens which did not die rapidly, the main post-mortem findings were cardiac hypertrophy and enlarged congested kidneys with marked glomerular hypertrophy which was presumably compensatory.

The significance of these findings is discussed.

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HAEMOLYTIC ANAEMIA WITH JAUNDICE IN SHEEP

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INTRODUCTION

This article describes two outbreaks of haemolytic anaemia with jaundice in half-bred hoggs grazing on (a) kale, (b) rape, which occurred on neighbouring farms in Berwickshire in September, 1951. In one case the sheep had been wholly on kale for a period of one month having previously grazed the usual ley pasture of grass and clover. The other sheep had been on rape for a similar period for part of each day only and had spent the rest of the time on a clover ley pasture.

RESULTS

Symptoms

The sixty hoggs on both farms were in fair condition when seen. About twenty of the kale-fed group had shown clinical signs of jaundice in varying degrees for two to three days previously, but only a few of the rape-fed sheep were affected. Seven of the former, and three of the latter, died. The others began to recover rapidly within a few days of the crisis after being removed from the kale. The worst affected sheep hung their heads in a dejected manner and stood with arched back and sunken eyes. Respiration and pulse rates were very fast. The visible mucous membranes were dark yellow-brown in colour and the skin was yellow. There was considerable diarrhoea. The temperatures varied between 104° F. and 105° F. One of the affected animals was seen to pass urine of a dark brown colour. Death occurred quickly in the more badly affected animals after a short period of coma.

Post-mortem Examination

One very badly affected animal was destroyed and immediately examined post-mortem. On opening the body the most striking feature was the deep yellow pigmentation of all the tissues and the dirty brown coloration of the blood. The liver was friable and bright yellow-brown in colour. The liver lobules were very distinct, their centres being deep red and their periphery orange in colour. Fibrosis was not present. The gall bladder was very distended. Both kidneys were embedded in very yellow fat and were black in colour. The cut surface showed that the black coloration extended

throughout both cortex and medulla; the tissue, however, was firm and of normal consistence unlike the kidney of enterotoxaemia. The spleen was small in size. The bladder was filled with clear urine of a dark brown colour due to the presence of haemoglobin. The thyroid glands appeared to be normal.

Bacteriological Examination

The usual methods of examination were carried out with negative results. No Leptospira were detected by dark ground illumination of urine, liver tissue and kidney tissue or by biological tests, and none were seen in sections of liver and kidney stained by Levaditi's silver impregnation method. Biological examination of the contents of various portions of the intestine gave no evidence of the presence of a toxin while cultural examination of the bowel contents showed coliform organisms to predominate. Bowel filtrates were tested for the presence of haemolysin with negative results.

Histological Examination

Sections of liver tissue showed a moderate degree of centrilobular necrosis with no cellular infiltration. The surviving polygonal cells of the liver showed considerable fatty change. The kidneys and other tissues of the body appeared normal.

Haematological Examination

Blood samples were obtained from three cases showing symptoms of varying severity and one case recovering from the crisis.

Cell counts

Affected sheep. The red blood cells were 3,600,000 to 4,200,000 per cu. mm.: reticulocytes (9 to 11 per cent.). The following changes were seen : anisocytosis, achromasia and polychromasia, punctate basophilia, Cabot's rings and Howell-Jolly bodies, reticulocytes and normoblasts. White blood cells were 700 to 900 per cu. mm.

One recovering sheep. The red blood cells were 8,100,000 : reticulocytes (12 per cent). All forms of regeneration were present. White blood cells were 3,000.

So far as could be determined the fragility of the red blood cells was within normal limits. No protozoan parasites of the blood could be demonstrated in smears stained by Giemsa.

Biochemical Investigation

It will be observed that in the case showing signs of recovery the haemoglobin content was normal whilst in the other three cases it was exceptionally low—3·6 g. The non-protein-nitrogen figures are all high compared with the normal value of 30 to 35 mg. The case with the highest blood total phosphate was the one termed "not so

	Haemo-globin g.%	*Non Protein Nitrogen mg./100 ml.*	*Total Phosphate mg./100 ml.*	*Inorganic Phosphate mg./100 ml.*	*Calcium mg./100 ml.*	*Magnesium mg./100 ml.*	*Van den Bergh units*
Recovering	11·3	62	8·2	3·0	11·5	2·09	neg.
Very ill	3·6	86	5·1	3·0	11·2	2·56	—
Not so ill	3·6	72	9·0	4·9	12·0	2·60	delayed 10 units
Worst case	3·6	100	5·0	2·5	11·5	2·08	delayed 5 units

ill" whilst the "recovering" case had still a very low inorganic phosphate. The "worst" case had the lowest phosphate, both organic and inorganic. The calcium and magnesium levels were normal.

The Van den Bergh test showed an indirect reaction in cases 3 and 4 with high values of 10 and 5 units respectively. The very high colour of the serum and plasma due to haemolysis made it impossible to read the test in case No. 2. The indirect reaction would tend to show that the polygonal cells were unable to cope with the pigment being manufactured by the reticulo-endothelial cells and that it was passing direct to the hepatic vein.

A liver tolerance test—the bromosulphalein test—was carried out on Case 4. The lamb weighed 100 lb. and 5 c.c. of bromosulphalein (5 mg./Kg.) was given. At 30 minutes after injection bromosulphalein was still present in the blood whereas in normal sheep it usually disappears entirely within 10 or 12 minutes. In case 4 it was absent from the blood 3 hours after injection. This would indicate a moderate degree of upset of liver metabolism.

The copper content of the liver from case 4 was 4,850 p.p.m. Cu in the completely ashed material. Since the liver contained 1·08 per cent ash and weighed 862 g. the total copper in the liver would be 180 p.p.m. on a dry matter basis.

DISCUSSION

Haemolytic jaundice of sheep has not been previously reported in Gt. Britain but numerous references have been made to it in other countries. Most of the work on the condition in sheep has been carried out by the Toxaemic Jaundice Investigation Committee of Australia (1949) and the conclusion reached was that symptoms of haemolytic anaemia with jaundice may arise under several conditions :—

(1) Chronic copper poisoning of sheep grazing copper-contamin- food.

(2) Sheep grazing *Heliotropium europaeum* which leads to liver damage in most of the grazing sheep and haemolytic anaemia in a few.

(3) Sheep grazing subterranean clover.

In these cases the haemolytic anaemia is due to the rapid discharge of copper into the blood stream from a liver having a high copper content. The copper content of the pasture in such cases is often within normal limits and the reason for the high copper content of the liver and blood is unknown. Bull (1951) states that the presence of haemoglobinuria in sheep is highly presumptive of chronic copper poisoning. If this is associated with central necrosis of the liver lobules, a premonitory rise in blood copper and a high copper content of the liver, then a diagnosis of chronic copper poisoning can be made.

In the present cases the total copper in the liver examined was 180 p.p.m. which is very much lower than the figures given by the Australian workers on Toxaemic Jaundice. Most of their cases had a liver copper concentration of over 1,500 p.p.m. on a dry matter, fat-free basis and a figure of 500 p.p.m. was considered to be relatively low. The value of 180 p.p.m. found in case 4, a half bred lowland sheep, is much above the average for Scottish hill sheep, the only type of sheep from which estimations of the normal copper content of the liver have been made. It is considered, however, on the Australian figures, that the present disease syndrome was not due to any form of copper poisoning.

Rose and Edgar (1936) stated that Toxaemic Jaundice of sheep in Australia is caused by the toxins of *Clostridium wechii* type A but further work has not confirmed their findings. There is, however, no doubt that the toxin of *Cl. welchii* type A produces in sheep a marked haemolytic anaemia (Gordon, Stewart, Holman and Taylor, 1940), but examination of the bowel contents in the present case did not show the presence of any type of toxin nor in fact was *Cl. welchii* cultured from the bowel.

Rosenberger (1943) and Cohrs (1943) have reported a haemolytic anaemia in cattle fed on kale to which they give the name 'kale anaemia' and their findings are very similar to our own although Rosenberger was unable to reproduce the condition in sheep. Rosenberger (1950) considers that kale anaemia in cattle is very similar to puerperal haemoglobinuria of cattle and our present findings also support that view.

It would seem that both haemolytic anaemia and fairly severe liver damage were present in our own cases as shown by the histological, haematological and biochemical examinations and by a liver function test. Similar findings to these have been described in postparturient red water of cattle (Hjärre, 1930) and the fact that the affected sheep showed very low inorganic phosphate levels is of interest as Stewart (1952) has found similar low phosphate

figures in cattle in postparturient red water. These findings differ from those of Rosenberger who reported normal phosphorus levels in his kale anaemia of cattle.

In Aberdeenshire, where a number of cases of puerperal haemoglobinuria of cattle occur every year, its cause is attributed to the feeding of swede turnips. In Canada, a similar disease with the same blood picture is said to be caused by sugar beet or fodder beet. Madsen and Neilson (1939) found that lucerne or byproducts of sugar beet caused a similar disease. In the present instance both kale and rape appear to have caused the disease syndrome.

SUMMARY

Two outbreaks of haemolytic jaundice amongst sheep grazing (a) kale (b) rape are described. The clinical, pathological and biochemical examinations suggest that the condition may be allied to puerperal haemoglobinuria of cattle.

ACKNOWLEDGMENT

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THE DETERMINATION OF ANTIBIOTIC LEVELS IN BLOOD AND IN MILK FOLLOWING PARENTERAL AND INTRAMAMMARY INJECTION

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INTRODUCTION

While considerable evidence has appeared in the literature concerning the retention of penicillin in the body fluids of man and animals following parenteral administration, newer antibiotics, less widely used for therapeutic purposes in animals, have not been examined so thoroughly. Many workers have investigated the retention of penicillin in the milk after intramammary infusion but there is little information concerning other antibiotics when employed in this way. It would seem important at this stage that the subject should be further explored in order to elucidate the question of dosage and intervals between administration for therapeutic use.

The object of the present study therefore was to determine the blood levels obtaining in animals following parenteral injection of aureomycin and streptomycin by various routes and to compare these with the levels of penicillin. A determination of the persistence of these three antibiotics in the milk of cows following intramammary infusion was also made and various techniques for their assay in serum and in milk were investigated.

When injected in an aqueous solution, penicillin is maintained only for a short period in the blood of cows, excretion being rapid in the urine and bile (Watts and McLeod, 1946; Slavin, 1946 ; Kelly and Bell, 1947 ; Morse, 1948). With regard to aureomycin, Larsen and Gilman (1950) have shown that it persists for at least 12 hours following the intravenous administration to cows of a dose of 11 mg./kg. body weight while McCollum and Doll (1951) found detectable blood levels for 20 hours after giving a similar dose to foals. In the case of streptomycin, Doll and Wallace (1948) showed that, following the intramuscular administration of a dose of 8.8 mg./kg. to sheep the antibiotic was present in the blood for 14 hours, but Hughes and Farmer (1950), using an intravenous dose of 10 mg./kg. found that it was present regularly only for 6 hours in the blood of calves and for 4 hours in cows.

Blood Serum Levels following Parenteral Injection

MATERIALS AND METHODS

Doses selected and routes of administration

In testing the therapeutic efficiency of certain antibiotics in mice, Edwards (1952) used a dose of 50 mg./kg., but it was found necessary to give this dose in divided amounts at regular intervals to control certain

infections. In the present investigation, therefore, a dose of 25 mg./kg. was employed in rabbits and goats. This represented the mean of the doses used to control experimental infections in mice. Previous studies on the retention of antibiotics in the bovine have been made with varying doses as has been described above. A dose of 11 mg./kg. (5 mg./lb.) was finally selected for the cow which is about half that used for smaller animals and accords with that generally recommended in the case of aureomycin. Solutions for injection were made in sterile distilled water. Penicillin was prepared from the sodium salt of penicillin G, streptomycin from the dihydrostreptomycin sulphate salt, and aureomycin from the crystalline hydrochloride with sodium glycinate added as buffer (the maximum solubility of aureomycin in solution was 5 per cent.).

After a preliminary trial in rabbits injected by the subcutaneous route, a comparison was made of the retention of the antibiotics in the blood of goats when injections were made by the subcutaneous and intramuscular routes. In lactating cows the intravenous and intramuscular routes were compared and a determination of the concentrations present in the milk made simultaneously.

Determination of antibiotic concentration in blood

Blood samples were taken aseptically at intervals into 1 oz. McCartney bottles. The blood clot was incubated for 10 minutes at 37°C. and then centrifuged ; the serum was collected and stored at −4°C. overnight and then tested for antibiotic content.

The following methods were used for assaying the concentration of each antibiotic :—

Penicillin. A serial dilution method in a liquid medium was used. In this test, 1 ml. of serum was serially diluted with an equal volume of 1 per cent. glucose broth in 3″ × ½″ sterile plugged tubes. A sensitive strain of *Str. agalactiae* was grown for 18 hours at 37°C., standardised to tube 1 on Brown's scale and a 1 in 4 dilution made in broth. One drop of this diluted culture was added to each tube in the series and one drop of a saturated solution of phenol red as indicator. The tubes were read after incubation for 24 hours at 37°C. and note taken of the end point i.e. of the highest dilution of serum which inhibited the growth of the test organism. At the same time the endpoint in a control series prepared from serum containing 1 μg./ml. of penicillin was determined; this end-point took place at a concentration of 0·03 to 0·06 μg./ml.

Aureomycin. A serial dilution technique based on that devised by Dornbush and Pelcak (1948) was employed. The same medium and technique as for penicillin assay were used but the test organism was *B. cereus* grown in broth and standardised to tube 2 on Brown's scale. Readings to determine the end-point of the serum dilution test series and of a control series prepared from serum containing 0·4 μg./ml. were made after 6 hours incubation at 37°C. This test gave detectable inhibition at a concentration between 0·006–0·01 μg./ml. aureomycin.

Streptomycin. A serial dilution method using the medium of May, Voureka and Fleming (1947) was employed and the test organism was Friedlander's bacillus (Klebsiella 41) standardised as for the streptococcus in penicillin assay. It was necessary to heat serum samples to 56°C. for 30 minutes in order to destroy normal inhibitors to the growth of the test organism present in unheated serum. Readings were made after 24 hours incuba-

tion at 37°C. when the inhibitory end-point in a control series usually occurred at a concentration of 0·08 μg./ml.

Rabbits RESULTS

Tests in rabbits were carried out in order to obtain preliminary information on the duration of the antibiotics in the blood after subcutaneous injection. The results obtained are shown in Table I.

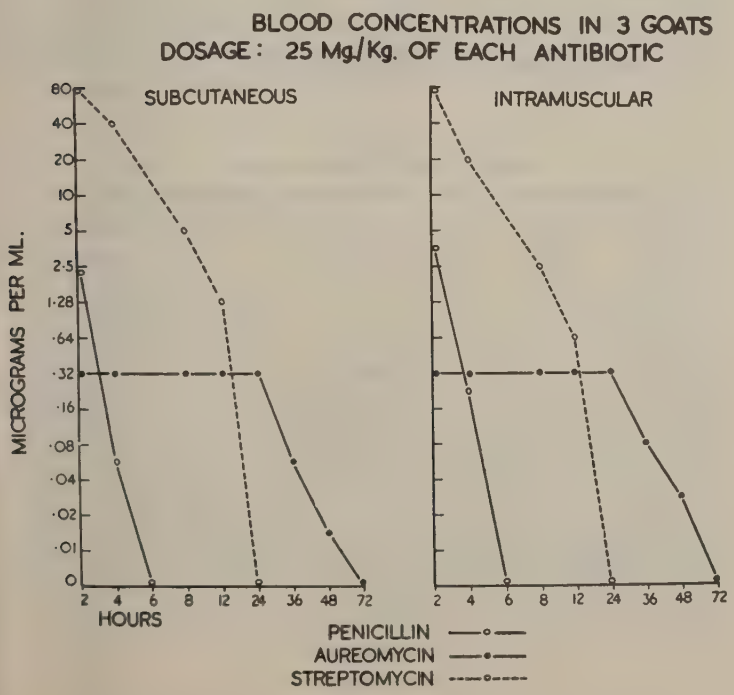
TABLE I

MEAN SERUM CONCENTRATIONS (μG./ML.) OF FOUR RABBITS INJECTED SUBCUTANEOUSLY WITH A DOSE OF 25 MG./KG.

Antibiotic	*Hours after injection*				
	2	4	8	12	24
Penicillin	0·5	0·12	0	—	—
Aureomycin ..	0·64	0·64	0·32	0·16	0·16
Streptomycin ..	40	20	1·28	0·64	0·32

There was a slight variation in the values obtained with different rabbits after injecting penicillin but this antibiotic was only present in the blood for 4 hours. Aureomycin and streptomycin on the other hand gave uniform values and persisted for 24 hours, which was the maximum period of sampling.

Fig. 1



Goats

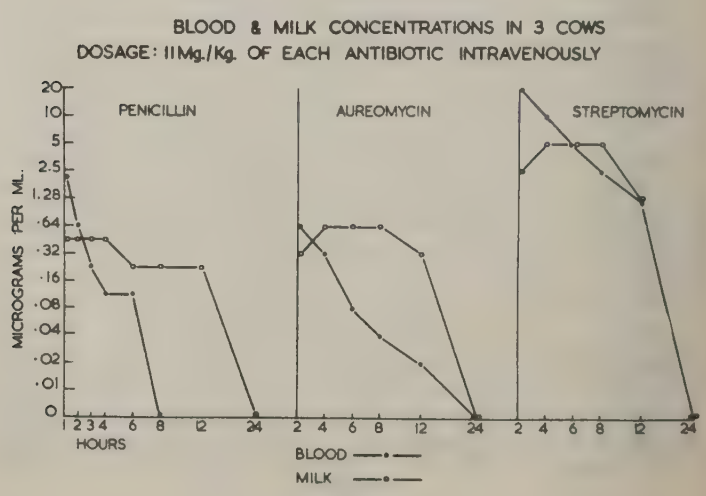
Goats of approximately 30 lbs. live weight were injected by the subcutaneous and intramuscular routes using the same dose (25 mg./kg.) of each antibiotic as was used in rabbits. Three goats were injected by each route and blood samples were taken at intervals up to 8 hours in the case of penicillin and up to 72 hours for the other two antibiotics.

The variation in concentration of antibiotic in the blood of the three goats injected by each route was small and the mean values obtained are shown in Fig. 1. It will be seen that there is a close similarity in the results obtained by the two routes with each of the three antibiotics. As with rabbits, penicillin was detected in the blood for 4 hours only after injection ; streptomycin persisted for 12 hours and aureomycin for 48 hours. The concentrations of aureomycin were relatively low throughout and remained constant at a mean value of 0·32 μg./ml. up to 24 hours, falling to 0·02 to 0·04 μg./ml. at 48 hours.

Cows (injected by the intravenous route)

Three lactating cows each giving approximately 2 gallons of milk daily were injected with a dose of 11 mg./kg. and the concentration of antibiotic determined in the serum and in a sample of the fore-milk taken at the same time. Each antibiotic was tested in turn on the same three cows. The mean concentrations observed in the blood and milk of the three cows are shown in Fig. 2.

Fig. 2



Penicillin was present in the blood for 6 hours, and a fairly constant level of 0·24 to 0·48 μg./ml. was maintained in the milk for 12 hours but was absent at 24 hours. Aureomycin remained in the blood for 12 hours only while a slightly higher concentration was found in the milk for the same time. Streptomycin showed a higher initial level than either penicillin or aureomycin in the blood and in the milk, beginning with 20 μg./ml. in the former and 2·5 μg./ml. in the latter and persisting for 12 hours.

Cows (injected by the intramuscular route)

Since, in practice, it is easier to administer antibiotics to the cow by the intramuscular rather than the intravenous route, it was of interest to study this route especially with a view to finding the antibiotic level present in the milk.

With penicillin and streptomycin the dose of 11 mg./kg. (5 to 7g.) dissolved in 50 ml. sterile distilled water was injected into the gluteal muscles of one leg. Three cows were injected with penicillin and streptomycin but only two with aureomycin since this antibiotic had proved to be irritant in a preliminary trial. The dose of aureomycin was dissolved in 100 ml. distilled water and injected into both hind legs.

The results in each of the three cows injected with penicillin and streptomycin respectively were closely alike and the mean concentrations are shown in Table 2 and Fig. 3 while those for the two cows injected with aureomycin are shown separately since there was a variation in the levels obtained.

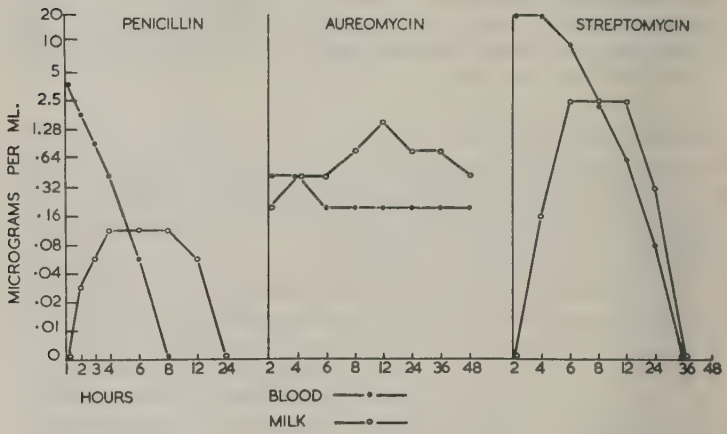
TABLE 2

MEAN CONCENTRATION OF ANTIBIOTICS IN BLOOD AND MILK OF COWS AFTER INTRAMUSCULAR INJECTION OF A DOSE OF 11 MG./KG.

Antibiotic	*Daily Milk Yield (lbs.)*	*Sample tested*	*Concentration (μg./ml.) in sample at intervals (hours)*									
			1	2	3	4	6	8	12	24	36	48
Penicillin	13—29	Blood	3·84	1·92	0·96	·48	·06	0	0	0	—	—
		Milk	0	·03	·06	·12	·12	·12	·06	0	—	—
Strepto-mycin	„	Blood	—	20·48	—	20·48	10·24	2·56	0·64	0·08	0	0
		Milk	—	0	—	0·16	2·56	2·56	2·56	0·32	0	0
Aureo-mycin	20	Blood	—	0·4	—	0·4	0·2	0·2	0·2	0·2	0·2	0·2
		Milk	—	0·2	—	0·4	0·4	0·8	1·6	0·8	0·8	0·4
	3	Blood	—	0·4	—	0·4	0·4	0·2	0·1	0·1	0·1	0·05
		Milk	—	3·2	—	3·2	3·2	6·4	6·4	6·4	3·2	3·2

Fig. 3

BLOOD AND MILK CONCENTRATIONS IN COWS
DOSAGE : 11 Mg./Kg. OF EACH ANTIBIOTIC INTRAMUSCULARLY



The results obtained with penicillin injected by this route were similar to those by the intravenous route although the concentrations found in the milk were not so high. While streptomycin persisted in the blood and milk for 24 hours, it did not appear in the milk until 4 hours after injection. Aureomycin presented a somewhat similar blood picture to that found in goats injected by the same route since it persisted in low concentration for at least 48 hours. The concentration of aureomycin in the milk of the cow yielding 2 gallons was slightly higher than in the blood, but in the low yielder it rose to a much higher level.

Although no irritation had been observed in goats following the injection of aureomycin intramuscularly, in cows it produced a local swelling and induration of the muscles persisting for 10 days.

Milk Levels Following Intramammary Infusion

Observations have been made by several workers on the residual concentrations established in the milk of the cow following infusion of aqueous solutions and oily suspensions of penicillin. The main interest regarding penicillin is in the residual concentration found in the milk following the use of different suspending vehicles.

The work now described is concerned with the infusion of the udders of lactating cows with antibiotics prepared in forms convenient for intramammary therapy and the subsequent determination of the concentration established in the milk. At the same time an enquiry has been made into the existing methods of antibiotic assay in milk and further techniques have been evolved.

MATERIALS AND METHODS

Pre-treatment of milk samples. Milk samples were taken with aseptic precautions into sterile 1 oz. McCartney bottles. About 10 ml. of the sample were then transferred to a sterile screw-capped container and immersed in a water bath at 62° C. for 30 minutes. Tests carried out on milk samples containing known concentrations of penicillin, aureomycin, and streptomycin showed that there was no deterioration in potency following such heat-treatment.

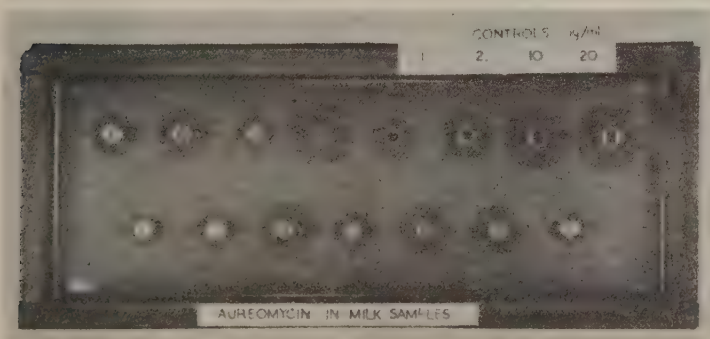
Antibiotic assay. Two methods have been studied for assaying antibiotics in milk namely the cylinder-cup plate method and the serial dilution method.

Plate method. This is a modification of methods previously described by Florey, Abraham, Chain, Fletcher, Gardner, Heatley and Jennings (1941) and by Heatley (1944).

Wooden rectangular frames, 12″ long by 4″ wide, placed between two glass plates are used instead of petri-dishes since they provide a relatively large surface allowing 16 assays to be made on one plate. In order to seal the frame to the glass plate 50 ml. agar of poor nutritive quality are poured into the plate and, when solidified, a further 50 ml. inoculated with the test organism poured on to the surface of the first layer. Sterile porcelain cylinder cups are then placed on the surface in two parallel rows, equal distances apart (about 1¼″). The cylinder cup is filled with the milk sample under test and the plate incubated at 37° C. for the required length of time. The ring diameter in a control series containing varying concentrations of antibiotic plotted against the log of the concentration gives a straight line and this is conveniently shown on semi-log paper.

The plate method was used for assaying penicillin in milk samples using agar seeded with a 1/500 dilution of a broth culture of a staphylococcus strain (N.C.T.C. 6571A) and the test read after 18 hours at 37° C. Contrary to the findings of Price, Randall and Welch (1948) this method was found to give satisfactory results in detecting aureomycin using *B.cereus* as the test organism since it was found that there was reasonable consistency in the variation in diameter of the diffusion zones given by increasing concentrations of aureomycin. This variation in the diameter of different concentrations in a control series and in milk samples under test is shown in Fig. 4.

Fig. 4



Aureomycin assay by the plate method.

The plate method has the advantage that a large number of samples can be tested more rapidly than by a serial dilution test and it is sufficiently sensitive for the detection of concentrations as low as 1 μg./ml. In our hands, it was not found suitable for streptomycin assay since this antibiotic did not produce consistent zones of inhibition.

Serial dilution. This method was employed as an alternative to the plate method and for checking milk samples with low concentrations of antibiotic i.e. below 1 μg./ml. Different methods have been described for conducting this test and previous workers have employed whey for assay purposes. Thus McCulloch, Kiser and Migaki (1949) prepared it by adding acid before aureomycin assay, while Hughes and Farmer (1950) added rennet before streptomycin assay. Both these methods were found to be time consuming and resulted in a loss of antibiotic content during the preparation of whey.

A dilution technique was finally employed in which 1 ml. of whole milk was serially diluted in the same media as described previously for the serum assay of each antibiotic and the technique of performing the tests was the same except that brom-cresol purple was used as an indicator. A similar technique using whole milk has been described recently by Schipper and Petersen (1951) for determining the aureomycin content of milk samples, but the basic medium in this case was methylene blue milk

Penicillin

RESULTS

A comparison was made of the retention of this antibiotic following the infusion of the quarters of lactating cows with the following products each in 100,000 unit doses :— (1) calcium penicillin G suspended in a 5 per cent beeswax arachis-oil base, (2) procaine penicillin G suspended in a 3 per cent. aluminium-stearate arachis-oil base.

Eight lactating cows with varying yields were selected. Four of them received the calcium salt and four the procaine salt on one occasion; injections were made into each quarter *via* the teat canal after the afternoon milking and assays of the fore-milk were made at intervals following injection. Seven days later, injections were repeated but each group of four cows received a different product from that infused on the previous occasion.

The results obtained (see Table 3) do not agree with those of Zweig (1949) who found that procaine penicillin could be detected in the milk at 72 hours irrespective of the milk yield of the cow whereas calcium penicillin remained for 48 hours. They accord, however, with those of Hughes, Christie and Farmer (1950) and Hignett (1950) in demonstrating that penicillin is not consistently present even 48 hours after the injection of either product and that calcium penicillin is retained more regularly than the procaine product at the 72nd hour. It will be seen that there is a relationship between persistence and concentration of penicillin in the milk and the yield of the cow, thus confirming the findings of Murnane (1945), Schalm and Casselberry (1946) who found that the concentration is inversely related to the milk yield.

TABLE 3

RESIDUAL CONCENTRATIONS (μG./ML.) PRESENT AT INTERVALS FOLLOWING THE INJECTION OF 100,000 UNITS OF PENICILLIN SUSPENDED IN TWO DIFFERENT BASES

Product used	*Daily Yield (lbs.)*	*Quarters injected*	24 *hours*		48 *hours*		60 *hours*		72 *hours*	
			Quarters positive	*Ave. conc.*	*Quarters positive*	*Ave. conc.*	*Quarters positive*	*Ave. conc.*	*Quarters positive*	*Ave. conc.*
Ca. penicillin in 5% wax-oil	> 10	16	16	13	13	0·54	10	0·13	10	0·1
	< 10	16	16	16	16	2	16	0·6	15	0·97
		32	32	14·6	29	1·4	26	0·4	25	0·62
Procaine penicillin in 3% aluminium stearate oil	> 10	16	16	15·6	12	1·6	7	0·3	5	0·1
	< 10	16	16	22·0	14	4	13	0·9	12	0·7
		32	32	18·9	26	2·8	20	0·67	17	0·54

Aureomycin.

Although this antibiotic has been shown to yield successful results in the therapy of udder infections due to both staphylococci and streptococci (McCulloch *et al*, 1949; Packer, 1950; Slanetz and Allen, 1950; Hulse, 1951), few data concerning its retention in the milk have been presented. It is available for intramammary injection in coilapsible tubes containing 210 mg. aureomycin hydrochloride in 7 grammes of a petrolatum base.

In order to determine the residual concentration following this dose, each quarter of five cows was injected after milking and fore-milk samples were tested at intervals for 5 days.

It will be seen (Table 4) that aureomycin could be recovered in the fore-milk for 5 days and that while there was variation in the level of individual quarters, there was little indication that such variation was related to the daily milk yield of the cows injected. These results agree fairly well with the levels obtained by McCulloch *et al* (1949) who only examined the milk for 72 hours.

Following the injection of the aureomycin into normal udders, the foremilk became abnormal and contained yellowish oily globules for 2 to 3 days as well as an increased cell count for 4 days. Since some of the material contained in the fore-milk appeared to consist of particles of the suspension injected, it was thought that this might lead to a false assessment of the concentration in the milk within the udder due to uneven distribution of the antibiotic.

In order to verify this point the concentration present in the foremilk of each of four cows was compared with that present in the mid-milk and strippings at the same milking. The cows were in-

jected in each quarter with 210 mg. aureomycin hydrochloride, and the concentration assayed in the three portions of milk on 6 successive days.

TABLE 4

AUREOMYCIN CONCENTRATION IN THE FORE-MILK AFTER INJECTING 210 MG. HYDROCHLORIDE INTO EACH QUARTER OF FIVE COWS

Cow	*Yield lbs.*	*Quarter*	*Residual concentration (μg./ml.)*				
			24 hours	*48 hours*	*72 hours*	*96 hours*	*120 hours*
1	34½	L.F.	10·0	1·5	0·6	0·36	0·4
		L.H.	10·0	0·9	0·3	0·28	0·25
		R.F.	18·0	3·0	1·0	0·6	0·25
		R.H.	15·0	3·0	1·0	0·28	0
2	26¼	L.F.	28·0	3·0	2·8	0·8	0·25
		L.H.	28·0	3·0	0·5	0·36	0·2
		R.F.	10·0	1·5	0·78	0·36	0·1
		R.H.	15·0	3·0	0·44	0·13	0·15
3	16¼	L.F.	2·7	0·8	0·3	0·13	
		L.H.	3·7	1·0	0·23	0·19	—
		R.F.	6·8	1·0	1·4	0·7	
		R.H.	3·4	0·8	0·45	0·32	
4	13	L.F.	10·0	3·4	5·0	1·2	
		L.H.	2·0	0·6	0·16	0·1	—
		R.F.	2·9	1·4	0·9	0·7	
		R.H.	1·6	0·6	0·36	0·13	
5	11¾	L.F.	10·0	3·0	1·0	0·36	0·1
		L.H.	15·0	10·0	2·0	1·0	0·32
		R.F.	40·0	15·0	6·0	2·5	0·6
		R.H.	20·0	4·5	1·4	0·6	0·18
Average concentration			12·6	3·0	1·3	0·6	0·25

It will be seen (Table 5) that aureomycin was distributed throughout the milk and that the highest concentration was usually present in the strippings. Low levels of the antibiotic were found for 6 days in the milk of the cow giving 25 lbs. of milk daily whereas in cows with higher yields the antibiotic did not persist as long.

Streptomycin

The use of this antibiotic for the treatment of mastitis caused by Gram-negative bacteria has been reported by Schalm (1948) but its retention in the milk was not investigated. In the present study, the milk of five cows was assayed at intervals after injection into each quarter of a dose of 250 mg. of dihydrostreptomycin sulphate suspended in oil with the addition of 3 per cent aluminium stearate.

The results (Table 6) indicate that streptomycin is not retained after two days in the fore-milk even in the quarters of cows giving a low milk yield.

TABLE 5

AUREOMYCIN CONCENTRATION IN SAMPLES OF FOREMILK, MIDMILK AND STRIPPINGS AFTER INJECTING 210 MG. HYDROCHLORIDE INTO EACH QUARTER OF FOUR COWS

Cow	*Milk Yield (lbs.)*	*Sample*	*Residual concentration (μg./ml.)*					
			24 hrs.	*48 hrs.*	*72 hrs.*	*96 hrs.*	*120 hrs.*	*144 hrs.*
1	57	Foremilk	16	2	0·5	·03	0	0
		Midmilk	8	2	0·5	0·5	0·1	0
		Strippings	64	8	2	2	1	0
2	53	Foremilk	2	2	0·25	0	0	0
		Midmilk	8	2	0·25	0	0	0
		Strippings	32	4	1	1	0·5	0
3	52	Foremilk	16	1	0·5	0	0·25	0
		Midmilk	8	2	0·25	0·25	0	0
		Strippings	32	4	1	1	0·5	0
4	25	Foremilk	64	16	8	4	2	1
		Midmilk	16	2	1	1	1	0·5
		Strippings	64	8	2	4	1	0·5

TABLE 6

STREPTOMYCIN CONCENTRATION IN THE FORE-MILK AFTER INJECTING 250 MG. DIHYDROSTREPTOMYCIN SULPHATE INTO EACH QUARTER OF FIVE COWS

Cow	*Yield (lbs.)*	*Quarter*	*Concentration of streptomycin (μg./ml.)*		
			24 hours	*48 hours*	*72 hours*
1	41	L.F.	40·9	0	0
		L.H.	163·8	1·28	0
		R.F.	10·24	0	0
		R.H.	10·24	0	0
2	31	L.F.	10·24	5·12	0
		L.H.	0·64	0·32	0
		R.F.	20·48	0·32	0
		R.H.	1·28	0	0
3	29	L.F.	80	10	0
		L.H.	20	2·5	0
		R.F.	40	5	0
		R.H.	20	2·5	0
4	15	L.F.	81·9	1·28	0
		L.H.	81·9	1·28	0
		R.F.	10·24	0·32	0
		R.H.	5·12	0·64	0
5	10	L.F.	160	10	0
		L.H.	80	0·6	0
		R.F.	80	< 0·3	0
		R.H.	80	< 0·3	0

Trussell and Stevenson (1949) showed that streptomycin persisted for 12 hours when injected in bougie form while Hughes and Farmer (1950) found its presence 24 hours after injection in the milk of only one cow out of three. It was observed in the present test that the streptomycin product injected did not cause any irritation of the mammary gland.

DISCUSSION

It is of considerable importance to study the pharmacology of antibiotics before they are applied for therapeutical purposes in clinical infections. Upon such knowledge a correct procedure regarding dosage and interval between injections is developed and the most economical use made of the antibiotic.

In the work reported the residual levels in the blood of penicillin, aureomycin and streptomycin have been compared when each antibiotic has been injected in aqueous solution. By incorporating antibiotics in oily bases or by using insoluble salts either in oil or in finely dispersed suspension, it is known that longer retentions may be obtained than those established by aqueous solutions (Doll and Dimock, 1946; Kelly and Bell, 1947; Morse, 1949; Miller, Russo and Scheidy, 1949; Maclay and Stannard, 1950). Unfortunately, the antibiotics are not all available in the same suspended form for comparative study and these may not always be suitable for parenteral use. Further, the incorporation of antibiotics in oily or other bases masks their true rate of dissociation and excretion. The results presented show that there is a close similarity in the respective blood levels attained following the parenteral administration of the three antibiotics by the three routes employed, but a longer retention of aureomycin and streptomycin following the intramuscular route than the intravenous route.

The longer persistence of aureomycin than penicillin in the body probably accounts for the greater therapeutic value of the former demonstrated in the treatment of experimental infections in mice. Thus Klein, Schorr, Tashman and Hunt (1950) have shown that a single dose of 1 mg. of aureomycin given intramuscularly was more effective than the same dose of penicillin in combating type 1 pneumococcus infection. Again Edwards (1952) showed that the same dose of aureomycin given subcutaneously gave better protection than penicillin against virulent streptococcal and staphylococcal infections in mice. To explain the advantage of aureomycin, Klein *et al* state that following its injection intramuscularly it remains at the site of injection and is only slowly released so that a sustained protective level is achieved. The intramuscular route has the disadvantage, so far as aureomycin is concerned, of producing marked irritation when doses of 5 g. are given to cows. On the other hand in goats, when a dose of 350 mg. was employed no such irritation was

seen, so that it would appear to be necessary to compute a lower dosage level for cattle when this route is employed.

The finding that all the antibiotics were present in the milk of injected cows requires further comment since not all workers agree regarding their passage into the mammary gland. Thus Barker and Dussault (1945) found that penicillin could not be detected in the milk of one cow when injected for 4 days intramuscularly with a total dosage of over 2 mega units. Again Watts and McLeod (1946) found no penicillin in the milk of 4 cows following injection of 1 mega unit in aqueous solution. Slavin (1949), on the other hand, showed the presence of penicillin in the milk of cows 1½ hours after intramuscular injection of 2 to 5 mega units while Welsh and Burkhart (1949) found that aureomycin appeared in milk soon after intravenous injection and persisted for 36 to 48 hours i.e. for about 12 hours longer than levels could be detected in the blood. This result has been confirmed by Larsen and Gilman (1950).

From the practical point of view the excretion of antibiotics in the milk is important since parenteral administration may assist the recovery of mastitis cases which are refractory to intramammary injection. It is clear from the present investigation that concentrations which may be regarded as bacteriostatic for the ordinary udder pathogens can be expected for at least twelve hours. A reasonable level is obtained within one hour following the intravenous route and a little later following the intramuscular route. This level remains fairly constant in spite of the removal of milk by milking 6 to 8 hours after a preceding morning injection. The next milking, 20 to 22 hours later, depresses the milk level, so that in order to maintain the concentration a second injection should be administered at the time of the second milking. Aureomycin may be found in the milk for at least 48 hours after intramuscular injection but, as pointed out earlier, there is objection to the use of this route of injection on account of the resulting irritation.

The results of direct infusion of antibiotics into the udder show that penicillin and streptomycin suspended in retaining bases are present in the milk for a period of 48 hours in most quarters. It follows therefore, that if successful therapy depends on maintaining a continuous antibiotic level, maximum recovery should attend re-injections at 48 hour intervals. The evidence presented by Hughes *et al* (1950) however, indicates that it is not necessary, at least in order to achieve successful results with *Str. agalactiae* infection, to maintain a continuous concentration of penicillin and that a system of interrupted therapy with re-injection after 72 hours can effect a 90 per cent. bacteriological cure. The greater persistence of aureomycin than the other antibiotics after intramammary injection accords with its longer retention in the blood following parenteral injection, and for practical application by this route re-injections can be made after an interval of 4 days.

SUMMARY

The persistence and levels of penicillin, aureomycin and streptomycin in the blood of rabbits, goats and cows, after injecting doses of 11 to 25 mg./kg. body weight by three different routes, have been investigated.

Penicillin was found for no longer than 6 hours while streptomycin concentrations were maintained for 12 hours with an extension at a low level for 24 hours when the intramuscular route was used in cows.

Aureomycin was retained for at least 48 hours when injected subcutaneously or intramuscularly but was eliminated within 24 hours after intravenous injection.

When injected parenterally the three antibiotics were excreted into the milk of the lactating cow and in the case of aureomycin and streptomycin were detected over the same period as their presence was found in the blood, but for a longer period in the case of penicillin.

The retention of these antibiotics in the milk when incorporated in suitable bases for direct intramammary infusion was studied.

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STREPTOCOCCAL ENDOCARDITIS IN A PIG FOLLOWING INTRAVENOUS INJECTION OF AN ORGANISM FROM A NATURAL CASE

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INTRODUCTION

While the common cause of bacterial endocarditis in the pig is *Erysipelothrix rhusiopathiae*, some cases are due to other organisms, including streptococci. We have attempted to reproduce lesions of endocarditis in pigs by injecting a streptococcus recovered from a vegetation on the heart valve from a natural case in a young pig. In accordance with many reports of the experimental production of endocarditis by streptococci in rabbits, it was thought that repeated intravenous injections of the organism would perhaps be required to cause the development of valve lesions and two pigs were therefore given a series of intravenous injections of the streptococcus concerned, while two other pigs were given single doses, with the idea that they would serve as controls. In the event, however lesions of frank endocarditis were found in one of this second pair of pigs, while the two pigs given repeated injections showed no macroscopic valvular lesions. One pig of each pair developed lesions of arthritis.

MATERIAL AND METHODS

The strain of streptococcus used produced alpha-haemolysis on ox-blood agar. It grew on plain agar at pH 7·2 and 9·6, but not on MacConkey agar. In plain broth, it gave a moderate turbidity and slight deposit, as it did in serum broth. The organism was not heat resistant (60° F, 15 minutes). It produced acid in glucose, sucrose, lactose, maltose and salicin, but not in mannite or raffinose. In litmus milk, it first produced reduction and then acidity. Indol was not formed. Starch was hydrolysed, but the sodium hippurate, æsculin and methylene blue reactions were all negative. Precipitin tests were negative with group sera A, B, C, D, G, L and M.

The inoculum consisted in each case of a saline suspension of the centrifuged deposit from a 48-hour culture in glucose-broth, grown at 37°C. Injections were made into the anterior vena cava, or, on occasions when difficulty was encountered in using this route, into an ear vein. For culture, blood was drawn from the anterior vena cava into tubes of serum broth or saponin broth, some tubes being incubated at 37°C, others being used to prepare poured plates. Blood was also taken for the estimation of haemoglobin content, haematocrit value, and total and differential white cell counts. Four litter-mate pigs, which were fourteen weeks old at the start of the experiment, were used.

RESULTS

Two of the four pigs, C.1 and C.2, were given a single intravenous injection of the streptococcus. C.1 showed at autopsy a mitral endocarditis, renal lesions and arthritis, while C.2 showed histological evidence of endocarditis. Two pigs, C.3 and C.4, were given a series of intravenous injections of the organism. C.3 developed arthritis, but neither pig developed endocarditis. No significant changes were found in the blood picture.

Single Intravenous Injection

Pig C.1

On November 19, 1951, this pig was given intravenously the centrifuged deposit of 100 ml. of a 48-hour glucose-broth culture, suspended in 10 ml. saline and estimated to contain about 24,000,000,000 organisms. Three days after the injection the pig was lame in the right hind-leg. Its temperature was 105° F. There was no discoloration of the skin. The right hock joint particularly appeared stiff, and the animal spared the limb as much as possible. It fed moderately well but was increasingly disinclined to move. When lying quietly, obvious abdominal respiratory movements were seen. When the pig was roused, it walked normally, but lay down again to rest as soon as it was left alone, and showed forced expiratory movements. No significant changes were recognised in the blood picture, but a positive blood culture was obtained 12 days before slaughter, a rich growth of alpha-haemolytic streptococci appearing in a poured saponin-broth plate.

This pig gradually fell behind the other experimental animals in rate of growth and by the time it was killed, eleven weeks later, it was in thin condition.

At post-mortem examination, lesions were found in the heart, joints and kidneys.

Heart. A clear straw-coloured fluid, containing a gelatinous clot, was present in the pericardial sac, and there was a slight roughness of the epicardium, particularly over the auricles and left ventricle. The mitral valve (fig. 1) showed rather firm, pale grey-yellow vegetations on the auricular surface, which extended over the edge of the valve onto the chordæ tendinæ, while small nodular lesions were present on the endocardium just below the valve. The bulk of the vegetations consisted of fibrous tissue, which was relatively poor in cells except just below the surface where a layer of thrombus, polymorphonuclears and bacteria was seen. Many gram-positive cocci were seen in smears and in sections of the surface of the vegetations (fig. 2). A rich, pure growth of an alpha-haemolytic streptococcus, resembling that recovered in blood culture twelve days previously, was obtained. No lesions were found in the right heart, nor in the anterior vena cava. Despite the size of the valve lesion, no excess fluid was present in the larger body cavities, the

liver and spleen showed no sign of chronic passive congestion, and the lungs showed merely some prominence of the veins running along the interlobular septa.

Joints. An excess of a turbid and blood-stained fluid was present in the right and left hip, left shoulder, and right and left elbow joints; in the two latter joints, a gelatinous fibrin clot was also present, and in the left joint many hæmorrhages occurred around the beak of the olecranon process. In sections of the synovial membrane of the right hip joint, the synovial cells appeared swollen and numerous, there was capillary congestion and inflammatory cells were present. An alpha-haemolytic streptococcus, similar to that recovered from the mitral valve, grew in culture from the left shoulder joint.

Kidneys. The cortical surfaces showed a heavy sprinkling with small red and grey points, which were also just visible on the cut cortical surface. In section (fig. 3) hyaline material was seen in the capsular spaces and in the lumen of the tubules. Some red blood cells were present in this hyaline material, and in some tubules casts of inflammatory cells were seen. Occasional tubules showed marked hyaline droplet degeneration of the epithelium. Scattered, slight focal interstitial accumulations of mononuclear and polymorphonuclear cells were present, and a number of isolated eosinophils were seen in glomeruli and interstitial tissue. No streptococci were obtained in culture from the kidneys.

Although the strain of streptococcus recovered from pig C.1, both from blood culture twelve days before slaughter and at autopsy, was biochemically similar to the injected strain, it differed in its growth in serum broth, leaving the supernatant fluid clear. Both the injected and the recovered strains gave positive slide agglutination with serum from the pig obtained on 10/12/51, 19/12/51 and 5/2/52, but gave negative results with serum taken on 19/11/51, at the start of the experiment. Owing to spontaneous agglutination of the strain recovered from the experimental pig when suspended in saline, it was not possible to secure clear-cut readings with tube agglutination tests. As far as they went, however, these results suggest that the recovered strain was the same as the one injected.

Pig C.2

This pig was given a similar dose to C.1 on 21st November, 1951. It grew well, showed no significant changes in its blood picture, gave no positive blood cultures and exhibited no macroscopic lesions when it was slaughtered on 8th February, 1952, eleven weeks after the intravenous injection of streptococci. A random section of the mitral valve revealed (fig. 4) a microscopic inflammatory focus immediately beneath an intact endothelium, comprising mainly small and medium-sized mononuclear and occasional polymorphonuclear cells. Similar cells were found in the depth of the tissue.

Repeated Intravenous Injections

Pig C.3

This pig was given intravenously on 21st November, 1951; the centrifuged deposit of 100 ml. of a 48 hour glucose-broth culture, suspended in 10 ml. saline; similar doses were injected on the 5th, 9th, 14th, 23rd and 28th days. The deposit from 400 ml. of culture was given on the 44th, 48th, 51st, 55th and 64th days, making a total of 11 doses in all. The animal was killed on the 29th January, 1952, five days after the last dose and ten weeks after the first dose. Blood cultures made before each injection were sterile and no changes of significance were noted in the blood picture. Eleven days before it was killed, this pig appeared very stiff in its limb joints, particularly in the right fore-leg, but small doses of potassium bromide in the food appeared to give relief.

At post-mortem examination, the heart valves appeared normal macroscopically, but in sections, polymorphonuclear cells were found discretely scattered in the connective tissue of the tricuspid valve. The other valves appeared normal in section. There was no obvious damage to the anterior vena cava at the site of the injections, and the kidneys appeared to be normal.

Some of the limb joints (right hip, right and left stifle, left hock, left elbow) contained a quantity of blood-stained fluid in which were a few flakes of fibrin; the synovial membranes showed a patchy reddening and there was some oedema around the joints. The presence of arthritis was confirmed histologically (figs. 5 and 6). An alpha-haemolytic streptococcus, with cultural characters similar to those of the injected strain, was recovered from the left stifle and left elbow joints.

Pig. C.4

This pig received a similar course of injections to C.3, starting on the 21st November, 1951, and lasting 28 days; it was killed on the 4th January, 1952, six weeks after the first injection and 16 days after the last. No significant changes were noted in the blood picture, but one blood culture taken on the 14th day yielded isolated colonies of an alpha-haemolytic streptococcus, which was, however, biochemically different from the injected strain.

No obvious lesions were detected in the heart, joints, kidneys or elsewhere at post-mortem examination, but a few discretely-scattered polymorphonuclears were present under the endothelium and in the tissue at the base of a heart valve. No streptococci were recovered in culture from any site.

DISCUSSION

In view of the importance of bacterial endocarditis in man, much work has been done on the experimental production of the condition. The animal chiefly used has been the rabbit, and a common method

of trying to produce valvular lesions has been to give intravenous injections, generally repeated, of streptococci, with or without previous measures to damage the endocardium. While endocarditis has often been produced in such experiments, there are certain difficulties in the interpretation of the results and in their application to the elucidation of the pathogenesis of the condition. For example, it is difficult to ascertain in the living animal when the lesions of endocarditis are developing; such findings as repeated positive blood cultures, cardiac murmurs, loss of weight, fever, and increased sedimentation rate, have been regarded as helpful. Further, if endocarditis should be found after a series of repeated injections, it is impossible to be certain whether all, or some, or only one, of the injections have been concerned in the production of the lesion. Again, many of the experiments have been made with strains of organisms foreign to the species of experimental animal used.

In the pig, endocarditis has been produced in experiments with *E. rhusiopathiae* (Collins and Goldie, 1940) and with streptococci (Hont and Banks, 1944). The latter organisms were used in experiments in Australia, endocarditis being found in a pig which had been given repeated intravenous injections of a mixture of young cultures of two strains recovered from natural cases of porcine endocarditis. It seems probable that the endocarditis present in one of our pigs (C.1) was produced as a result of the single intravenous injection of the streptococcus used, although the difference of growth in serum broth between the strain injected and the strain recovered by blood culture and from the mitral valve lesion raises some doubt. It is known that spontaneous cases of streptococcal endocarditis do occur in pigs (Kernkamp, 1941), and we cannot eliminate the possibility that this too was a spontaneous case. However, this difficulty must be present in experiments where an organism causing spontaneous disease is used in the homologous species, and we feel that our results suggest that the pig, which has many merits as an experimental animal, might prove suitable for a study of endocarditis.

SUMMARY

Two pigs were given a single intravenous dose of an alpha-haemolytic streptococcus recovered from a natural case of porcine endocarditis, and two were given repeated intravenous doses. Lesions of endocarditis were found macroscopically in the first, and histologically in the second pig of the pair receiving the single doses. Arthritis was found in one pig of each pair.

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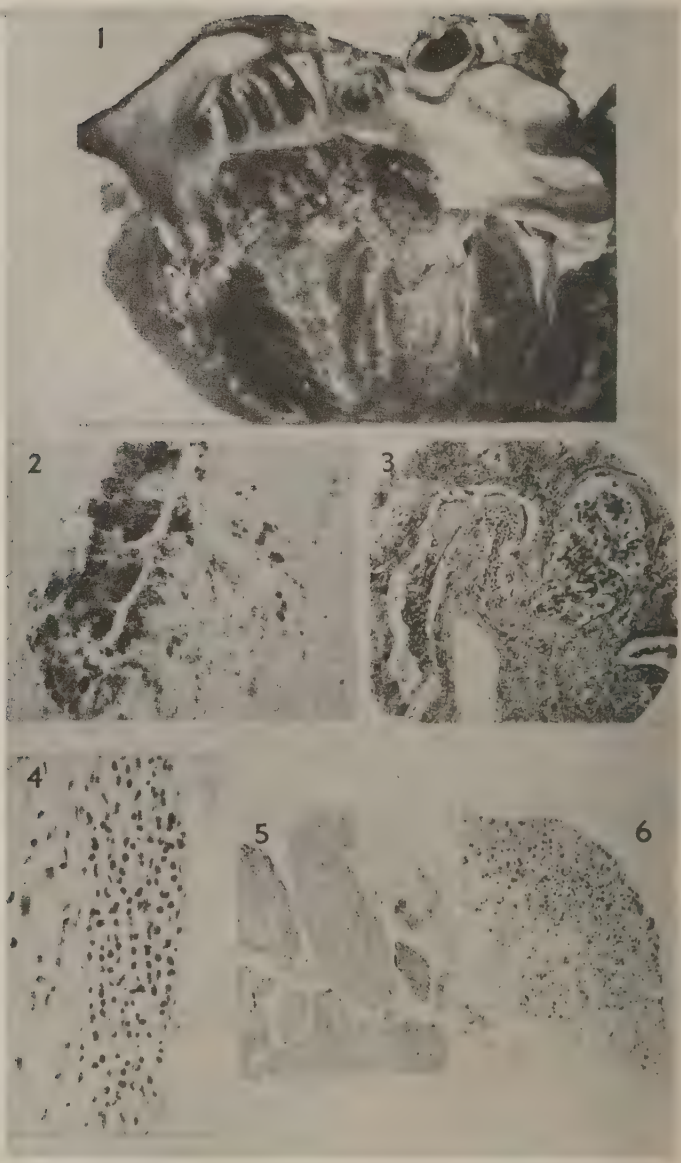

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LEGENDS TO ILLUSTRATIONS

Fig. 1. Mitral valve lesion of pig C.1, showing vegetations on auricular surface of valve and spreading over edge and down chordæ tendinæ. × 2/3.

Fig. 2. Small cleft in surface of heart valve vegetation of pig C.1, showing numerous cocci, scattered and in dense clumps. Gram. × 240.

Fig. 3. Kidney of pig C.1, showing a glomerulus and tubule containing hyaline material and many red blood cells. Haematoxylin and eosin. × 120.

Fig. 4. Mitral valve of pig C.2, showing subendocardial focus of inflammatory cells, including small and larger mononuclear cells and some polymorphonuclears. Haematoxylin and eosin. × 240.

Fig. 5. Left hock joint of pig C.3, showing cellularity of synovial membrane and fibrinous exudate. Haematoxylin and eosin. × 35.

Fig. 6. Left hock joint of pig C.3, showing increase in number and swelling of synovial cells, and inflammatory cell accumulation. Haematoxylin and eosin. × 160.